DOPAMINERGIC NEURONS IN THE VENTRAL TEGMENTAL AREA: ROLE OF L-TYPE CALCIUM CHANNELS IN FIRING REGULATION

YUDAN LIU


CENTRE FOR NFLD. STUDIES
MEMORIAL UNIVERSITY
OF NEWFOUNDLAND

Dopaminergic Neurons in the Ventral Tegmental Area: Role of L-type Calcium Channels in Firing Regulation

by

Yudan Liu

A thesis submitted to the

School of Graduate Studies

in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

Division of Biomedical Sciences

Faculty of Medicine

Memorial University of Newfoundland

April, 2009

St. John's Newfoundland

Abstract

Dopaminergic projections from the ventral tegmental area constitute the mesolimbocortical system that underlies drug abuse and schizophrenia, primarily as the result of increased dopamine transmission. Essentially, more intense spiking releases more dopamine at the terminal, such as fast single spike firing or burst firing. L-type calcium channels are expressed in dopaminergic neurons, however their role in regulating firing frequency and modes remains unknown. In this thesis, I combined patch clamp recording, western blotting and L-type calcium channel transgenic mice to examine the effects of L-type calcium channels on the firing behavior of dopaminergic neurons in brain slices. Results revealed that calcium influx through L-type calcium channels following FPL64176 or (S)-(-)-Bay K8644 application induced burst firing independent of dopamine, glutamate or calcium from internal stores. The burst firing induced as such was completely blocked by the substrate site protein kinase C (PKC) inhibitor chelerythrine but not by the diacylglycerol site inhibitor calphostin C. Western blotting analysis showed that FPL64176 and (S)-(-)-Bay K8644 increased the cleavage of PKC to generate protein kinase M (PKM) and the specific calpain inhibitor MDL28170 blocked this increase. Prevention of PKM production by inhibiting calpain or depleting PKC blocked burst firing induction whereas direct loading of purified PKM into cells induced burst firing. Activation of the NMDA type glutamate or cholinergic receptors known to induce burst firing increased PKM expression. These results indicate that calcium influx through L-type calcium channels activates a calcium-dependent protease that cleaves

PKC to generate constitutively active and labile PKM resulting in burst firing of dopaminergic cells (Chapter 2). Next, I examined the role of the L-type calcium channel and PKC in firing responses to carbachol, NMDA or AMPA. All three ligands induced a reversible increase in firing, however, only the carbachol-induced increase was attenuated by the PKC inhibitors chelerythrine and GF 109203X. The L-type calcium channel blocker nifedipine partially blocked carbachol-induced excitation similar to the PKC inhibitors. PKC inhibition and L-type calcium channel blockade did not significantly alter NMDA- or AMPA-induced excitation. Concurrent blockade of PKC and L-type calcium channels with chelerythrine and nifedipine did not additionally suppress carbachol-induced excitation indicating they were sequential events in the same signaling pathway. Furthermore, preincubation with the PKC inhibitor GF 109203X reduced the carbachol-induced increase in nifedipine-sensitive high-voltage gated calcium currents. These results indicate that cholinergic activation enhances PKC activity, which in turn facilitates L-type calcium channel opening to excite dopaminergic cells (Chapter 3). Then, I examined which subtype of L-type calcium channels was involved in firing activities in L-type transgenic mice that have a mutant dihydropyridine (DHP) site. Single spike firing was inhibited to the same extent by the DHP site blocker nifedipine in both $Ca_v1.2DHP^{(+/+)}$ and $Ca_v1.2DHP^{(-/-)}$ mice. The DHP site opener (S)-(-)-Bay K8644 and the non-DHP opener FPL64176 induced bursting of dopaminergic cells in $Ca_v1.2DHP^{(-/-)}$ mice similarly and the DHP blocker nifedipine blocked bursting induced by either agent. Since Cav1.2 and Cav1.3 are the only subtypes expressed in dopaminergic cells, these

results underscore the importance of $Ca_V1.3$ L-type calcium channels in single spike and burst firing of these cells (Chapter 4).

Acknowledgements

I wish to thank my supervisor Dr. Xihua Chen, who has provided me the opportunity to participate in the Neuroscience program in Memorial University and to complete my Ph.D. thesis work in his lab; who has opened a door to me toward a scientific world and guided me patiently and persistently; who has taught me the scientific thinking and how to find a useful way in complex scientific and reality world. St. John's is my first place to visit far away from my home country. His wife, Jieying Xiong and he, have provided countless valuable help in my life like a mother and a father.

I wish to thank Dr. Jules Dore, who has provided me an opportunity and resources to learn Western blotting and PCR genotyping, with which my thesis work is more complete.

I wish to thank Dr. Gilbert Kirouac, my committee member, who has provided me useful suggestions on my thesis work and my future plans.

I wish to thank my parents Min Wang and Hongmin Liu, who have not only nurtured me but also taught me many things in my life; who always encourage and support me to pursue my dreams in any time; whose endless love has always accompanied me.

I wish to thank my extended family, my grandpa and grandma, my aunts and uncles, my cousins, especially Xin Chen, and my sister Yongdan Liu, who has provided endless love and countless support to me in my life.

I wish to thank my lovely Chinese friends, Zhili Kang, Dr. Jinguo Wang, Sa Li, Lan Chen, Dr. Yaping Li, Dr. Xuhan Liu, Dr. Ningyuan Feng, and Dr. Chunming Dai, who always has shared laughs and tears with me and held my hands whenever I needed. Without them, I could not survive in the tough Ph.D. study far away from my home country.

I wish to thank my lovely Canadian friends, Heather Moore and Meghan Harding, who has provided me their love and care; who has shared with me the excitement of life; who has always made me laugh; who has taught me a lot of English and things about western life. Without them, my life in Canada could not be this colorful.

I wish to thank the School of Graduate Studies, Faculty of Medicine and my supervisor Dr. Xihua Chen for having provided me the financial support throughout my program, and all the faculty members and students in the Neuroscience program who have given me the help and support.

Table of Contents

Chapter 4 $Ca_v1.3$ L-type Calcium Channels Mediate Single Spike and Burst Firing of VTA DA Neurons in Mice

List of Table

List of Figures

List of Abbreviations

ACSF, artificial cerebrospinal fluid;

AChR, acetylcholine receptor;

ACh, acetylcholine;

AHP, afterhyperpolarization potential;

CaMKII, calcium/calmodulin-dependent protein kinase II;

CV, coefficient of variance;

CPA, cyclopiazonic acid;

DA, dopamine;

DAG, diacylglycerol;

DAT, dopamine transporter;

DHP, dihydropyridine;

EGFP, enhanced green fluorescent protein;

GAD, glutamic acid decarboxylase;

GIRK channel, G protein-activated inwardly rectifying K^+ channel;

HVA, high-voltage-activated;

I_h, hyperpolarization-activated current;

ISIs, interspike intervals;

LDTg, laterodorsal tegmental nuclei;

LSD, least significant difference method;

mGluRs, metabotropic glutamate receptors;

NAc, nucleus accumbens;

PKC, protein kinase C;

PKM, protein kinase M;

PKA, protein kinase A;

PMA, phorbol 12-myristate 13-acetate;

PMSF, phenylmethylsulfanyl fluoride;

PPTg, pedunculopontine tegmental nuclei;

PVDF, polyvinylidene fluoride;

RMP, resting membrane potential;

SK channel, calcium-activated small conductance potassium channel;

SERCA, sarco-endoreticulum Ca^{2+}-dependent ATPase;

SEM, standard errors of the mean;

SNpc, substantia nigra pars compacta;

TH, tyrosine hydroxylase;

TRP channel, transient receptor potential channel;

TTX, tetrodotoxin;

VTA, ventral tegmental area;

WT, wild type.

Chapter 1 Introduction and Overview

The central nervous system allows us to process environmental stimulation into conscious percepts and organize our actions in response to a specific task. This calls for two fundamental requirements of neurons as functional units of the central nervous system: they need to be able to receive inputs and they need to transform these inputs into an output. The neuronal output is electrical in nature which is referred to as an action potential. Action potentials are the language of neurons, like the notes of music, the dots of Braille and the signs of a sign language. Action potentials are all-or-none: a neuron either fires an action potential or nothing at all. Because of this feature, an individual action potential has a very limited capacity for information coding. However, a train of action potentials occurring at different frequencies and in different temporal patterns codes for all our different brain functions such as movement, learning and motivation. This is analogous to the huge amount of words formed with a limited number of letters.

Dopaminergic (DA) systems originating from the ventral tegmental area (VTA) play a vital role in reward processing, leading to addiction and psychosis primarily as the result of increased DA transmission. Since the frequency and temporal pattern of action potential trains code for all neuronal information, the regulation of the firing behavior of DA neurons is essential for their roles in reward processing and disease states. This chapter gives a general review on (1) the functional role of the DA system, (2) the modulation of firing activities and its functional importance, (3) the functional

significance of L-type calcium channels in the DA systems and (4) the hypotheses of my projects.

1.1 The central DA systems and their functions

The Swedish pharmacologist Arvid Carlsson performed a series of pioneering studies during the lat 1950's, which showed that DA is an important transmitter in the brain (Carlsson, et al., 1957;Carlsson, et al., 1958). It was previously believed that DA was only a precursor of another transmitter, noradrenaline. Arvid Carlsson developed an assay that made it possible to measure tissue levels of DA with high sensitivity. He found that DA was concentrated in specific parts of the brain, which led him to the conclusion that DA is a transmitter in its own right. Since this first discovery that dopamine is an independent transmitter in the brain and further studies and conclusion that DA plays an important role in Parkinson's disease by Arvid Carlsson (Carlsson, 1988), the central DA systems and their roles in physiological functions have intrigued neuroscientists for several decades.

1.1.1 Brief anatomy

It is known that 70-80% of the DA neurons in the brain are mainly located in three distinct areas in the midbrain: the VTA (A10), substantia nigra pars compacta (SNpc, A9) and the retrorubral field (A8). The rest of the DA neurons are primarily located in the

hypothalamus. DA neurons in areas of the midbrain are organized in two major systems: the mesostriatal and mesolimbocortical systems (Bjorklund and Dunnett, 2007). The mesostriatal system mostly originates in the SNpc and projects to the dorsal striatum, such as the putamen and caudate nucleus (Anden, et al., 1964). The mesolimbocortical system (Figure 1.1) originates in the VTA and mostly projects to the prefrontal cortex and ventral striatum such as the nucleus accumbens (NAc) (Anden, et al., 1965;Ungerstedt, 1971). The VTA is an area without clear anatomical boundaries and with neurons scattered diffusely. In general, the level of the caudal tip of the mammillary tract is considered as the rostral border and the medial edge of the medial terminal nucleus of the accessory optic tract as the lateral border. There are some subregions in the VTA, such as the parabrachial pigmental area, which have different neuronal compositions and densities (Nair-Roberts, et al., 2008;Yamaguchi, et al., 2007). With the use of neurochemical markers, for example, the vesicular glutamate transporter 2 for glutamatergic neurons, tyrosine hydroxylase (TH) for DAergic neurons, and glutamic acid decarboxylase (GAD) for GABAergic neurons, three types of neurons have been found in the VTA: 60% are DA neurons; 35% are GABAergic neurons; only 2-3% are glutamatergic neurons highly enriched in medial parts of rostral VTA (Kosaka, et al., 1987;Nair-Roberts, et al., 2008;Yamaguchi, et al., 2007).

1.1.2 Connectivity of the VTA

A variety of transmitter terminals exist in the VTA including GABAergic, glutamatergic, cholinergic, serotoninergic and noradrenergic. Other amino acid transmitters such as glycine and neuropeptides such as neurotensin, substance P, opioids and orexin also have effects on DA cells in the VTA. This section only covers the important excitatory and inhibitory transmitters: glutamatergic, GABAergic and cholinergic inputs (Figure 1.2). The glutamatergic afferents arise mainly from the prefrontal cortex, the laterodorsal (LDTg) and pedunculopontine (PPTg) tegmental nuclei (Carr and Sesack, 1999;Georges and Aston-Jones, 2001;Semba and Fibiger, 1992). In the meantime, there is also a possible local glutamatergic input since a small portion of cells utilizing glutamate as a neurotransmitter have been reported to be present in the VTA (Nair-Roberts, et al., 2008;Yamaguchi, et al., 2007). Moreover, it has been suggested that glutamate can also be co-localized in VTA DA neurons and DA neurons can use glutamate as a cotransmitter (Chuhma, et al., 2004; Mendez, et al., 2008; Descarries, et al., 2008; Lavin, et al., 2005). The GABAergic inputs to the VTA DA neurons mostly originate in the ventral striatum and pallidum (Groenewegen, et al., 1993;Smith and Bolam, 1990;Somogyi, et al., 1981) and also from the GABAergic interneuron within the VTA (Bayer and Pickel, 1991;Kosaka, et al., 1987). The cholinergic inputs arise in the LDTg and PPTg (Beninato and Spencer, 1987;Bolam, et al., 1991;Clarke, et al., 1987;Cornwall, et al., 1990;Satoh and Fibiger, 1986). These pontine nuclei thus provide both glutamatergic and cholinergic innervations to the DA neurons (Lavoie and Parent,

1994a;b) and send an extensive projection to the midbrain, where the terminals closely surround the soma and proximal dendrites of the DA neurons (Lavoie and Parent, 1994c).

The DA neurons of the VTA project to limbic and cortical areas along mesolimbic and mesocortical pathways (Figure 1.1). The limbic terminal areas include the ventral striatum, the ventro-medial part of the head of the caudate-putamen, amygdala and hippocampus (Bjorklund and Dunnett, 2007). The cortical DA innervation is largely confined to areas of the frontal, cingulate and entorhinal cortex (Bjorklund and Dunnett, 2007). Based on connectivity and morphological features, the VTA DA neurons can be separated into a dorsal and a ventral tier. The dorsal tier includes cells located in the dorsal aspect of the VTA innervating ventral striatal, limbic and cortical areas as well as the matrix compartment of the dorsal striatum (Bjorklund and Dunnett, 2007;Gerfen, et al., 1987;Lynd-Balta and Haber, 1994). These cells are round or fusiform in shape, are characteristically calbindin-positive, and express relatively low levels of the dopamine transporter (DAT). The ventral tier comprises a sheet of more densely packed, angular cells located in the ventral parts of VTA (Gerfen, et al., 1987;Prensa and Parent, 2001). These cells are calbindin-negative, express higher levels of DAT and are mostly immunopositive for the ion channel protein GIRK2 (G protein-activated inwardly rectifying K^+ channels). The ventral tier neurons project to the striatum where they innervate the patch compartment.

1.1.3 Function of the VTA

The VTA DA system plays an important role in the regulation of cognitive functions (Le Moal and Simon, 1991), memory (Lisman and Grace, 2005), emotion (Laviolette, 2007;Pezze and Feldon, 2004), and reward-related behaviors (Koob, et al., 1998;Robbins and Everitt, 1999) through their networks with the limbic or cortical areas (Figure 1.1, 1.2, 1.3). Specifically, distorted DA output from the VTA has been implicated in diseases such as schizophrenia (Carlsson, 1988;Laviolette, 2007) and drug dependence (Di Chiara, 1995;Di Chiara and Imperato, 1988).

The prefrontal projections of the DA neurons from the VTA are considered to modulate neuronal activities related to cognitive functions such as working memory, planning and execution of behavior, and maintenance of focused attention (Le Moal and Simon, 1991). Local injection of DA D_1 receptor antagonists, such as SCH 23390, into prefrontal cortex impairs the adequate performance of working memory tasks (Sawaguchi and Goldman-Rakic, 1994), while it also has been shown that activation of DA neurons in the VTA by stimulating the mu-opioid receptor with enkaphalin produces a dose-dependent impairment of working memory tasks, which is restored by blocking the DA D_1 receptor with the antagonist SCH 23390 in prefrontal cortex (Romanides, et al., 1999). Therefore, it has been proposed that working memory function is optimized when DA D_1 occupancy is within a critical range of an inverted U-shaped function (Arnsten and Li, 2005;Floresco and Phillips, 2001;Zahrt, et al., 1997). Increased DA efflux during

working memory tasks has been observed in the primate prefrontal cortex as well (Watanabe, et al., 1997). In addition, reward-related stimuli also increase DA activities in this region (Taber and Fibiger, 1997).

The VTA DA neurons are profoundly implicated in the abuse of many drugs, e.g. cocaine and nicotine, which are known to directly or indirectly cause an increased transmission of DA in terminal areas such as NAc and prefrontal area (Di Chiara, 1995;Di Chiara and Imperato, 1988), inducing euphoria and reinforcement of drug seeking behavior. For example, in animals that are trained to self-administer cocaine, infusion of a DA receptor antagonist into the terminal field NAc dose-dependently decreases the frequency and total amount of the drug self-administration (Bachtell, et al., 2005). Selective destruction of the mesolimbic DA system using the neural toxin 6-hydroxydopamine inhibits self-administration of drugs of abuse in rats (Corrigall, et al., 1992;Roberts, et al., 1980). Rats can also learn to electrically self-stimulate the DA neurons of VTA (Kilpatrick, et al., 2000). Psychostimulants primarily enhance DA transmission through blocking DA reuptake or facilitating reverse transport in the terminal (Bradberry and Roth, 1989), but they also involve the cell bodies by synaptic receptor activation, D_2 autoreceptor-mediated inhibition or other compensatory changes associated with chronic use (Bunney and Aghajanian, 1977;Paladini, et al., 2001;Paladini and Williams, 2004). For instance, in several *in vitro* studies nicotine has been shown to excite DA neurons (Calabresi, et al., 1989;Picciotto, et al., 1998;Pidoplichko, et al., 1997) whereas opiates have been shown to inhibit the GABAergic inputs to DA neurons

(Johnson and North, 1992a), leading to a disinhibition of the DA neurons, and therefore both nicotine and opiates excite the DA neurons in the VTA to enhance the DA transmission in terminal fields although by different mechanisms.

Schizophrenia is caused by multiple factors involving multiple transmitter systems, however, it is strongly believed that the central DA system plays a significant role in this disease (Carlsson, 1988;Laviolette, 2007). Drugs which directly or indirectly facilitate DA neurotransmission in the brain, e.g. amphetamine or L-DOPA, can precipitate or aggravate psychosis. All effective neuroleptic drugs used for the treatment of schizophrenia share anti-DA activity and the clinical potency of an antipsychotic drug to suppress positive symptoms of schizophrenia ,such as delusions and hallucinations, and the ability of the drug to block D_2 receptors are strongly correlated (Seeman, et al., 1976). However, the atypical antipsychotic drug clozapine, with lower occupancy of D_2 receptors and higher affinity to block D_1 and D_4 receptors, produces a better relief of the negative symptoms of schizophrenia, such as blunted affect and emotion, alogia and avolition (Seeman, 1990). Therefore, schizophrenia has been thought to be associated with a differential dysfunction of the DA systems with hyperactivity in some areas like the NAc inducing the positive symptoms, and hypofunction in others like prefrontal area resulting in the negative symptoms (Goto, et al., 2007). However, a new version of the DA hypothesis of schizophrenia was proposed by Howes and Kapur recently (Howes and Kapur, 2009), who thought that the hypothesis of subcortical hyperdopaminergia with prefrontal hypodopaminergia focused too narrowly on DA itself, conflated psychosis and

schizophrenia, and predated advances in the genetics, molecular biology, and imaging research in schizophrenia. The new hypothesis proposed by Howes and Kapur has 4 distinctive components: " multiple risk factors, such as genes, stress and trauma, drug use, or pregnancy and obstetric complications, interact to result in DA dysregulation – the final common pathway to increase presynaptic striatal DA function to induce the psychosis in schizophrenia; the locus of DA dysregulation moves from being primarily at the D_2 receptor level to being at the presynaptic DA control level; DA dysregulation is linked to 'psychosis' rather than schizophrenia; and the DA dysregulation is hypothesized to alter the appraisal of stimuli, perhaps through a process of aberrant salience." The new hypothesis has one major implication for treatment approaches. Current treatment are acting downstream of the critical neurotransmitter abnormality. Future drug development and research into etiopathogenesis should focus on identifying and manipulating the upstream factors that converge on the DA funnel point.

1.2 The modulation of firing activities and its functional importance

Action potential firing is the sole neuronal output, therefore, the firing behavior of neurons is important for their functions.

1.2.1 Firing activities and DA transmission

The functional importance of the DA system in the VTA is closely related to changes in DA transmission. DA release is a spike-dependent process like that of other transmitters: a higher frequency and longer duration of spiking increases DA release (Bean and Roth, 1991). The level of DA at the synapse depends not only on the rate at which it is released, but also on how efficiently it is reuptaken into the terminals. Reduced reuptake, as in the case of psychostimulants, markedly boosts DA concentration in the synapse (Horn, 1990). Excitatory synapses on DA terminals also promote DA release (Sharma and Vijayaraghavan, 2003). Besides the actions at the terminal, the cell body of DA cells in the VTA regulates DA output by changing firing rates and modes and by responding to terminal as well as somatic autoreceptors (Bunney, et al., 1973;Cubeddu and Hoffmann, 1982).

Firing patterns, as well as firing rates, play an important role in DA release. DA neurons exhibit two common firing patterns: tonic spiking and bursting (Figure 1.4) (Grace and Bunney, 1984a;b). Tonic spiking refers to firing at a regular or irregular frequency that is generally lower than that of burst firing. Burst firing refers to a cluster of spikes appearing on a slow undulation of the membrane potential. The frequency of firing within a burst is irregular and a burst is usually followed by a pronounced post-burst hyperpolarization. Based on the measurement of extracellular DA concentrations *in vivo* in VTA terminal sites, burst firing is shown to release more DA from the terminal than

the same overall frequency but with a constant inter-stimulus interval (Gonon and Buda, 1985;Manley, et al., 1992). This is probably due to Ca^{2+} accumulation in the pre-synaptic terminal (Grace, 2000;Suaud-Chagny, et al., 1992), or to saturation of the DA reuptake mechanisms during burst firing (Chergui, et al., 1994). The accumulation of DA in the synaptic cleft will have two significant effects: more pronounced postsynaptic effects and stronger autoinhibition of DA cell bodies in the VTA by activation of the D_2 autoreceptors because of the higher dendritic DA release due to an increase firing (Grace, 2000). Activation of release-modulating DA autoreceptors results in a feedback decrease in the release of DA (Wolf and Roth, 1987). These findings indicate that the activity of DA cell bodies can have far-reaching effects on DA transmission.

In addition, the importance of firing activities is also reflected in behavior. Freeman and Bunney have shown that burst firing in behaving rats is associated with the presentation of a novel visual or auditory stimulus (Freeman and Bunney, 1987). Studies in the monkey by Schultz et al (Schultz, 1997;1998;Tobler, et al., 2005) have analyzed the role of midbrain DA neurons in reward-related tasks and demonstrated that the DA neurons are, indeed, activated by a primary reward and especially if it is unexpected. If the reward is coupled to a conditioning signal (visual or auditory), the regular firing of the DA neurons will switch to burst firing or increase their firing rates from the moment of appearance of the actual reward to the instant when the conditioned signal is delivered. Moreover, if the conditioning stimulus is not followed by the expected reward the DA neurons decrease their firing. The DA neurons can thus detect deviations from the

expected reward so that a positive signal is delivered to the brain if the reward is better than expected, no change if the reward is just as expected and negative if less than expected.

In summary, firing activities in the DA neurons signal the occurrence of novel and rewarding stimuli, which implies that highly processed information is being passed to the DA neurons where the changes of firing activities have effects on projecting areas related to differing functions.

1.2.2 Firing regulation

Although it is widely recognized that firing activities of DA neurons in VTA are important for their function, how firing activities of DA neurons are regulated still remains to be fully understood. Up to the present, it has been thought that the regulation of firing activities would result from the interplay of excitatory and inhibitory mechanisms at the synaptic level, as well as from discrete intrinsic membrane properties at the cellular level. Two aspects of firing activities, firing rate and firing pattern, will be discussed below.

1.2.2.1 Extrinsic factors--excitatory and inhibitory synaptic inputs

DA neurons exhibit a highly regular pacemaker-like firing pattern *in vitro*, while bursting is more frequent *in vivo* (Grenhoff, et al., 1988), suggesting that the firing activities of these cells are strongly under the control of afferent inputs. DA neurons in the VTA receive intense inputs from a variety of origins. This section will only cover the important excitatory and inhibitory transmitters because they play a major role in modulating DA neuronal activity.

1.2.2.1.1 Glutamatergic input

As mentioned in section 1.1.2, DA neurons in the midbrain majorly receive direct or indirect glutamatergic inputs from different structures such as the prefrontal cortex, PPTg and LDTg (Carr and Sesack, 1999;Georges and Aston-Jones, 2001;Semba and Fibiger, 1992). Glutamatergic input from the prefrontal cortex, one of the major excitatory inputs, was shown to synapse onto DA neurons that loop-back to the prefrontal cortex, but not to those projecting to the NAc (Carr and Sesack, 1999;2000a;b). It is the input from other structures such as the LDTg that is likely to synapse onto DA neurons projecting to the NAc (Di Loreto, et al., 1992;Forster and Blaha, 2000;Omelchenko and Sesack, 2005;Sesack, et al., 2003).

Electrical stimulation of the medial prefrontal cortex enhances DA neuronal activity and produces burst firing (Gariano and Groves, 1988;Tong, et al., 1996b). Inactivation of this projection by cooling of the cortex or administration of glutamate receptor antagonists (Tong, et al., 1996a) reduces the firing rates and bursting, replacing it with pacemaker-like activity. Activation of the PPTg also enhances VTA firing activity and induces burst firing of DA cells (Lokwan, et al., 1999). Pressure-injected glutamate in the VTA stimulates the firing rate of DA cells and enhances extracellular DA concentration in projecting areas such as the NAc (Suaud-Chagny, et al., 1992). The effects of glutamate are due to three general groups of glutamate receptors that are expressed on DA neurons: AMPA, NMDA ionotropic and metabotropic receptors.

The major effects of glutamatergic input to DA neurons are mediated by AMPA and NMDA ionotropic receptors (Mathon, et al., 2003;Overton and Clark, 1997;Wang and French, 1993a;b). *In vivo* studies have shown that application of both AMPA and NMDA receptor agonists via microiontophoresis increases the firing rate of spontaneously active DA neurons. Both AMPA and NMDA receptor activation increases bursting activity in the SNpc, but the bursting activity in the VTA appears to be independent of AMPA receptors (Chergui, et al., 1993;Christoffersen and Meltzer, 1995;Overton and Clark, 1991;Zhang, et al., 1994). Studies in brain slices also show the excitatory effect of these receptors on DA neurons. Both application of AMPA and NMDA evokes an inward current and increases neuronal firing in a dose-dependent manner (Mereuri, et al., 1992b;Wang and French, 1993a;b;Wu, et al., 1994;Wu and Johnson, 1996). However, the

effects of a low dose of glutamate ($\leq$ 30 μM) on firing rate and membrane potential are mostly due to its actions at the NMDA receptors as they can be blocked by the noncompetitive NMDA blocker, phencyclindine, and the selective competitive NMDA receptor antagonist CGS 19755 (Wang and French, 1993b). NMDA receptor activation has been shown to induce burst firing under some conditions, particularly in the presence of hyperpolarizing currents or when calcium-activated potassium currents are blocked by apamin (Johnson and Seutin, 1997;Johnson and Wu, 2004;Seutin, et al., 1993). The mechanism underlying NMDA-induced burst firing can involve different ion species. For example, it is reported that Na^+ influx via NMDA-gated channels and the subsequent extrusion of Na^+ by the ouabain-sensitive electrogenic Na^+ pump comprises the full cycle of bursting (Johnson, et al., 1992). In summary, these findings underline the important excitatory role of AMPA and NMDA glutamate receptor activation in DA neuron regulation.

The metabotropic glutamate receptors (mGluRs) play a bidirectional role, either excitatory or inhibitory, on DA neurons depending on how the receptors are activated. Activation of the group 1 mGluRs located on DA neurons via synaptically-released glutamate, produces inhibitory postsynaptic currents and mediates a slow inhibition of DA neurons (Fiorillo and Williams, 1998). These inhibitory currents are a consequence of mobilization of intracellular calcium stores, which in turn activates inhibitory calcium-dependent potassium currents. However, with prolonged activation, the inhibitory response produced by mGluR activation desensitizes. Therefore, continuous

activation of these receptors induces a slowly developing sodium-dependent excitation, which can lead to an increase in impulse activity of DA cells (Fiorillo and Williams, 1998;Meltzer, et al., 1997;Mercuri, et al., 1993;Zheng and Johnson, 2002). It has also been suggested that activation of mGluRs modifies DA neuron activity indirectly by acting on presynaptic glutamatergic terminals so as to modify glutamate release. As for the post-synaptic effects, they can also be either stimulatory or inhibitory (according to the level of activation of the phospholipase C pathway) and produce, respectively, enhanced or reduced NMDA-mediated excitatory currents in post-synaptic cells (Bonci, et al., 1997;Gereau and Conn, 1995;Herrero, et al., 1992). On a similar line, at high doses, bath application of glutamate to isolated DA neurons has been shown to enhance spontaneous firing, but also to temporarily inhibit firing through two distinct calcium-dependent mechanisms: via activation of NMDA and AMPA receptors, or mGluRs (Kim, et al., 2004). Recently, it has also been shown that the action of mGluRs is linked to activation of transient receptor potential (TRP) channels because extracellular application of the non-selective TRP channel blocker SKF96365, flufenamic acid and ruthenium red causes reversible inhibition of mGluR1-activated EPSCs (Bengtson, et al., 2004). The group 1 mGluRs have been shown to play a role in the regulation of firing mode of midbrain DA neurons, especially in the SNpc (Meltzer, et al., 1997). The group 1 mGluR agonist increases the frequency of burst in vivo in the SNpc. Selective group 1 mGluR agonist (s)-3, 5-dihydroxyphenylglycine in the presence of tubocurarine or apamine, both of which are blockers of SK channels, can transform single spike firing to bursting in SNpc DA cells (Prisco, et al., 2002). Furthurmore, the mGluR antagonist

a-methyl-4-carboxyphenyl glycine [(s)-MCPG] blocks the post-burst hyperpolarization in bursting induced by repetitive extracellular stimulation or iontophoretic application of aspartate in midbrain DA slices (Morikawa, et al., 2003).

Together, these findings indicate that bursting may be ascribed, at least in part, to a complex combination of glutamate receptors. Activation of these receptors can exert both excitatory and inhibitory roles, either directly, or indirectly, by modifying glutamate release or the activity of other channels.

1.2.2.1.2 GABAergic inputs

GABA, the major inhibitory neurotransmitter, is responsible for most synaptically-induced direct inhibition of DA neuron activity. As described in section 1.1.2, the VTA receives a major GABAergic input originating in the NAc (Figure 1.2, 1.3) (Groenewegen, et al., 1993;Smith and Bolam, 1990;Somogyi, et al., 1981). Another important GABAergic input arises from local GABA interneurons in the midbrain (Bayer and Pickel, 1991;Kosaka, et al., 1987). Picrotoxin injected into the VTA to block $GABA_A$ receptors reduces ethanol intake of the alcohol-preferring rat (Nowak, et al., 1998). Microinjection of the $GABA_B$ receptor agonist, baclofen, into the VTA reduces the rewarding effect of morphine (Tsuji, et al., 1996). The cellular basis of these observed behaviors may be attributed to GABAergic influences on firing activities of DA cells in the VTA.

GABA afferents from the striatal complex play an important role in certain conditions, such as after the administration of psychostimulant drugs (Einhorn, et al., 1988;Pitts, et al., 1993). Hemitransection of this pathway fails to modify basal impulse activity in VTA DA neurons (Einhorn, et al., 1988;Pitts, et al., 1993;Pucak and Grace, 1994). After the injection of kainic acid into the dorsal striatum, there is a transient decrease in the percentage of activity of DA cells due to depolarization inactivation (Braszko, et al., 1981). The injection of kynurenic acid, the glutamate receptor antagonist, to the NAc produce a transient decrease in firing rate and burst firing of VTA DA neurons (Floresco, et al., 2001).

The presence of GABA neurons within the VTA (Figure 1.3) increases the complexity of the GABA effects and explains many biphasic effects produced by stimulation of brain structures projecting to the midbrain, or by systemic pharmacological treatments. Thus, stimulation of afferents onto DA neurons can have an initial response that is immediately followed by an opposite effect (Grace and Bunney, 1985;Overton, et al., 1996;Tong, et al., 1996b). This is because afferents can form synapses onto both DA and GABA neurons. Synapses onto DA neurons may produce direct neuronal inhibition, while synapses onto GABA neurons may produce indirect excitation of DA neurons by inhibiting local GABA interneurons because DA cells receive tonic inhibition from the interneurons. It has been shown experimentally that $GABA_A$-mediated synaptic responses in DA neurons exist tonically and can be prevented by tetrodotoxin (TTX) that blocks

spontaneous action potential firing of GABA interneurons in the slice (Johnson and North, 1992a). In addition, GABAergic synapses also exist on presynaptic terminals, indirectly affecting the DA neurons' excitability. For example, activation of $GABA_B$ receptors on presynaptic glutamatergic nerve terminals suppresses excitatory transmission onto GABA interneurons, relieving the tonic inhibition exerted by those neurons on DA cells (Wu, et al., 1999). Due to this anatomical arrangement, systemic administration of GABAergic drugs can have a paradoxical excitatory effect on DA neurons (Grace and Bunney, 1979;Waszczak and Walters, 1980).

The effects of GABA are due to two general groups of receptors that are expressed on DA neurons: $GABA_A$ ionotropic and $GABA_B$ metabotropic receptors. Local microiontophoretic application of the $GABA_A$ receptor agonist muscimol induces a clear-cut inhibition of DA neurons on a fast time scale (Erhardt and Engberg, 2000). On the other hand, the selective $GABA_A$ receptor antagonists bicuculline, gabazine and picrotoxin enhance both firing and bursting activity. Studies show that the $GABA_A$ receptor agonists modulate DA neuron activity by opening ionotropic receptors which directly allow chloride influx, thereby hyperpolarizing the cell membrane (Johnson and North, 1992b;Sugita, et al., 1992).

In contrast to $GABA_A$ receptors, $GABA_B$ receptors mediate inhibition of midbrain DA neurons on a slower time course. Local administration of the $GABA_B$ receptor agonist baclofen reduces firing and burst firing and converts NMDA-induced burst firing

to a single-spike firing pattern in DA neurons (Erhardt, et al., 1998;Erhardt, et al., 2002). Conversely, application of $GABA_B$ receptor antagonists increases DA firing and bursting, and prevents the effects produced by $GABA_B$ receptor activation (Chen, et al., 2005;Engberg and Nissbrandt, 1993;Erhardt, et al., 1998;Paladini and Tepper, 1999). Experiments using brain slices show that $GABA_B$ receptor agonists modulate the activity of DA neurons by activating $G_{i/o}$ proteins, the $\beta\gamma$ dimer dissociates from the heterotrimeric protein and binds to GIRK channels, which are differentially expressed by DA and non-DA neurons within the VTA (Cruz, et al., 2004). The consequent opening of these channels allows potassium outflow, which hyperpolarizes the cell membrane thereby inhibiting neuronal activity (Cruz, et al., 2004;Watts, et al., 1996).

1.2.2.1.3 Cholinergic input

The VTA has a high density of cholinergic terminals, indicating that it receives a substantial cholinergic input (Henderson and Sherriff, 1991). As detailed in section 1.1.2, the VTA receives bilateral innervation from PPTg and LDTg (Figure 1.2, 1.3), regions rich in cholinergic neurons (Beninato and Spencer, 1987;Bolam, et al., 1991;Clarke, et al., 1987;Cornwall, et al., 1990;Satoh and Fibiger, 1986).

Cholinergic input has been shown to play a vital role in regulating the firing activities of VTA DA neurons. Electrical stimulation of the PPTg produces an increase in the number of neurons firing in a bursting pattern, and this effect is produced only in

neurons that were already firing; PPTg inputs do not appear to be able to recruit inactive neurons or increase the firing rate of active neurons (Floresco, et al., 2003). In addition, electrical stimulation of LDTg results in a prolonged increase in DA release in NAc (Forster, et al., 2002) and chemical stimulation of the LDTg by infusion of a glutamate agonist or a muscarinic antagonist increases the number of active neurons in the VTA which is blocked by local application of the nicotinic receptor antagonist, mecamylamine. Interestingly, the inactivation of the LDTg by the administration of GABA agonists produces a pronounced decrease in the burst firing of DA neurons in the VTA that is not reversed by PPTg stimulation (Lodge and Grace, 2006). During LDTg inactivation, VTA neurons fire tonically in a very regular pattern, resembling the pacemaker firing pattern observed *in vitro* (Grace and Onn, 1989). The effects of cholinergic input are due to two general groups of receptors: nicotinic and muscarinic acetylcholine receptors (AChR). It has been shown that neurons in the VTA express somatic and dendritic nicotinic (Clarke and Pert, 1985;Clarke, et al., 1985;Sorenson, et al., 1998) and muscarinic (Nastuk and Graybiel, 1991) AChRs.

Nicotinic receptors are crucial in the initial fast response to acetylcholine (ACh) and mediate addiction (Balfour, et al., 2000). Acute systemic administration of nicotine increases intracranial self-stimulation rates (Druhan, et al., 1989) and locomotor activity in rats (Picciotto, 1998), while infusion of a nicotinic antagonist in the VTA results in a significant reduction in nicotine self-administration (Corrigall, et al., 1994) and in nicotine-induced locomotor activity (Louis and Clarke, 1998). On the same line, nicotine,

administered systemically *in vivo*, increases the firing rates and burst firing activity of VTA DA neurons associated with an increased release of DA in the NAc that is blocked by local application of the nicotinic receptor antagonist mecamylamine into the VTA, but not into the NAc (Grenhoff, et al., 1986;Nisell, et al., 1994). Infusion of nicotine into the VTA produces in DA neurons either a decrease in the input resistance and a transient depolarization of the membrane that results in the generation of action potentials, or an increase in the firing rate if the neuron was already firing and is followed by a rapid desensitization (Calabresi, et al., 1989;Pidoplichko, et al., 1997;Sorenson, et al., 1998). Furthermore, nicotinic receptors are shown to mediate presynaptic effects by enhancing glutamate release in experiments which exhibit that nicotine increases evoked and spontaneous excitatory synaptic currents in the VTA DA neurons (Dani, et al., 2001;Mansvelder and McGehee, 2000).

The muscarinic receptors are responsible for the prolonged response to ACh in DA cells (Lacey, et al., 1990) and are implicated in reward processing, e.g. eating and drinking. For example, infusion of the non-selective muscarinic receptor antagonist atropine into the VTA can stop self-stimulation and reduce food intake in rats (Rada, et al., 2000). *In vitro*, activation of postsynaptic M_1-like muscarinic receptors evokes a slow depolarization with an increase in firing rate and burst firing (Lacey, et al., 1990;Zhang, et al., 2005). Besides direct excitatory postsynaptic effects through M_1 and M_5 subtypes, muscarinic agonists can potently increase firing rates by reducing GABAergic transmission through presynaptic M_3 receptors (Grillner, et al., 1999). In addition,

muscarine is able to decrease after-hyperpolarizations which would facilitate firing at higher frequencies and possibly bursting (Scroggs, et al., 2001). Muscarinic receptors are shown to be coupled to G_q or $G_{i/o}$, activating phospholipase C leading to the hydrolysis of phosphoinositol or inhibiting adenylyl cyclase so as to have effects on different ion channels (Caulfield, 1993;Hulme, et al., 1990).

Several mechanisms have been proposed to explain how the activation by ACh might facilitate burst firing, including an increase in the level of intracellular calcium mediated by influx through NMDA receptors (Kitai, et al., 1999), activation of L-type calcium channels (Zhang, et al., 2005) and the mobilization of calcium from internal stores. All of these support the idea that the switch from single spike firing to burst firing following the activation of AChRs is a calcium-dependent mechanism.

In summary, cholinergic neurons of the brainstem regulate the firing rate of midbrain DA neurons and initiate their burst firing.

1.2.2.2 Intrinsic membrane properties

Numerous intrinsic membrane properties regulate the firing activity of DA neurons. This part will consider some of the most important ones, such as the DA autoreceptors, the afterhyperpolarization potential (AHP) and a hyperpolarization-activated current (I_h).

Voltage-gated Ca^{2+} channels, especially L-type calcium channels, will be described in 1.3.

1.2.2.2.1 DA autoreceptors

DA autoreceptors play an important role in providing an efficient negative feedback mechanism. These receptors are in the D_2/D_3 DA receptor family and are located in axon terminals and the somatodendritic region of VTA DA neurons (Clark and Chiodo, 1988;Mercuri, et al., 1992a). They are activated by synaptically and somatodendritically-released DA (Beart, et al., 1979;Cheramy, et al., 1981;Kalivas and Duffy, 1991) to reduce DA neuron activity by activating GIRK channels presumably via G protein $\beta\gamma$ subunits that are liberated by receptor activation of $G\alpha_{i/o}$ proteins. This produces a hyperpolarization in the cell body to inhibit firing (Figure 1.5) thus causing the terminals to reduce DA release (Davila, et al., 2003;Innis and Aghajanian, 1987;Lacey, et al., 1987;Mercuri, et al., 1992a;Williams and Lacey, 1988). Recent evidence shows that DA release depends on neuronal depolarization and this exocytotic release can directly inhibit neuron excitability (Beckstead, et al., 2004). Furthermore, the sensitivity of autoreceptors affects how the cell fires: fast-firing DA neurons have sub-sensitive impulse-regulating autoreceptors, conversely, slow-firing neurons exhibit greater sensitivity of these receptors (Marinelli and White, 2000;White and Wang, 1984) as revealed by the dose-dependent neuronal inhibition following local administration of D_2/D_3 receptor agonists into the VTA (Akaoka, et al., 1992;Gariano, et al., 1989;Pucak

and Grace, 1994). This effect is independent of differences in neuronal inputs because slow-firing cells are more sensitive to autoreceptor-mediated inhibition even when they are driven to a faster rate by local application of glutamate (White and Wang, 1984). Overall, these findings suggest that the functional state of somatodendritic DA autoreceptors mediates the activity of DA neurons.

1.2.2.2.2 Calcium-dependent potassium channels

Potassium channels come in many forms; both voltage-gated and calcium-activated small conductance potassium (SK) channels are implicated in midbrain DA neuron firing. Intracellular injection of the potassium channel blocker TEA increases the firing rate of DA neurons and produces burst-like activity (Grace and Bunney, 1984a). In addition, it has been shown that voltage-gated A-type potassium channels play a key role in modulating the pacemaker activity of DA neurons by combining real-time single-cell RT-PCR with slice patch clamp electrophysiology (Liss, et al., 2001). This pacemaker activity is related to A-type potassium channel density and the number of subunits that make up the potassium channel, indicating that both the amount and the type of gene expression are important in regulating DA neuron firing.

SK channels are found in DA neurons, where they contribute to the membrane AHP that follows each action potential by inducing an outward current after an individual action potential and thereby suppress repetitive firing at high frequencies. They have also

been proposed as the ionic basis for post-burst hyperpolarization in which a series of individual AHPs after a train of action potentials are summated to terminate the burst. These channels have slow dynamics to allow such a summation and are reported to be largely responsible for modulating burst firing activity (Overton and Clark, 1997). For example, regularly spiking DA cells in the VTA that display small AHPs respond to orexin A with bursting (Korotkova, et al., 2003).

Because SK channels are selectively permeable to potassium, and open when the intracellular calcium concentration is elevated, they are very sensitive to fluctuations of internal calcium concentrations. An increase in intracellular calcium activates SK channels, whereas a reduction in intracellular calcium prevents their activation. Thus, reducing the intracellular concentration of calcium, by intracellular injection of the calcium chelator EGTA, attenuates the AHP that follows a spike train (Grace and Bunney, 1984b) and increases DA neuronal firing (Grace and Bunney, 1984a). However, application of intracellular EGTA does not produce bursting because this manipulation not only inhibits SK channel activation, but also lowers intracellular calcium concentrations and prevents calcium influx from initiating burst firing. Physiologically, SK channels are activated by rises in intracellular calcium that can be produced by changes in calcium release from intracellular stores (Seutin, et al., 2000) such as those occurring after activation of metabotropic glutamate receptors (Morikawa, et al., 2000). Recently, it was shown that SK channels can also be activated by the calcium influx produced by activation of T-type calcium channels (Wolfart and Roeper, 2002). Because

of this, activation of T-type calcium channels can inhibit, rather than increase, DA neuron activity like bursting.

SK family channels that mediate AHP are described as SK1, SK2 and SK3 (Kohler, et al., 1996). SK2 expression is not as extensive as SK3 which is highly expressed in the VTA while SK1 is not expressed at all (Stocker and Pedarzani, 2000). SK2 and SK3 channels are very sensitive to the calcium-activated SK channel blocker apamin. Application of very low doses of apamin to DA neurons *in vitro* has been shown to interrupt pacemaker activity of DA neurons in the SNpc (Wolfart, et al., 2001), suggesting that these channels could play a role in modulating DA neuron pacemaker activity. Most importantly, application of apamin to midbrain DA neurons facilitates bursting activity produced by application of excitatory amino acids (Johnson and Seutin, 1997;Seutin, et al., 1993) and can be sufficient to produce burst firing in the tissue slice preparation (Ping and Shepard, 1996;Shepard and Bunney, 1991). Burst events produced by apamin consist of long bursts, two to tens of spikes per burst, which resemble natural bursting. The transition between the slow irregular firing and bursts is also controlled by fluctuations in membrane potential, which are modulated by SK channels (Amini, et al., 1999;Wilson and Callaway, 2000). Among SK channels, the SK3 subtype is expressed ubiquitously in midbrain DA neurons. In addition, its expression in these cells is either exclusive with respect to the other SK subtypes, or at least fourfold higher (Wolfart, et al., 2001), suggesting that it is the SK3 subtype that plays a major role in modulating burst firing activity in DA neurons.

1.2.2.2.3 I_h channels

I_h channels are largely expressed by DA neurons (Neuhoff, et al., 2002). As their name implies, these channels are activated when the neuron is hyperpolarized beyond -50 to -70 mV; their opening allows the inward flow of Na^+ and K^+, which produces membrane depolarization (Frere, et al., 2004). After hyperpolarizing steps, this is visible as a gradual increase in inward current when measured in voltage-clamp mode (Figure 1.5B), and as a typical "sag" when recorded in current-clamp (Figure 1.5C); in fact, the presence of this current or sag is often used as a "marker" for DA neurons recorded in the tissue slice (Grace and Onn, 1989;Johnson and North, 1992b;Mercuri, et al., 1995;Neuhoff, et al., 2002). I_h has been suggested to regulate low frequency pacemaker activity, however only a subset of SNpc neurons show altered pacemaker frequency in the presence of the I_h channel inhibitor ZD-7288; therefore, it is debatable whether the expression of this current regulates the overall firing pattern of most midbrain DA neurons (Mercuri, et al., 1995;Neuhoff, et al., 2002;Seutin, et al., 2001). I_h does however, regulate the amplitude and duration of the AHP that follows action potentials in all subtypes of midbrain DA neurons with a possible function of the integration of inhibitory input to these cells (Neuhoff, et al., 2002).

1.3 Role of L-type calcium channels in DA neurons

L-type calcium channels are one class of the voltage-gated calcium channels which consist of L-, N-, P/Q-, T- and R-type calcium channels, all of which are present in DA neurons (Cardozo and Bean, 1995;Takada, et al., 2001). Influx of calcium through calcium channels depolarizes the neuronal membrane, which can lead to cell firing. In midbrain DA neurons, intracellular injection of calcium in high concentrations *in vivo* increases burst firing whereas the calcium chelator EGTA reduces the depolarization that initiates bursting and thus also greatly reduces bursting (Grace and Bunney, 1984a). Although it is known that calcium regulates neuronal firing, it is unclear what channel calcium uses to enter the cell to mediate this action. *In vitro* studies show that calcium channel blockers for N- and P/Q-type channels reduce the membrane depolarization that initiates bursting but their contribution to the total depolarization is far less than the L-type calcium channels and may not play a large role in DA neuron firing (Durante, et al., 2004). In contrast, activation of T-type calcium channels decreases neuronal firing by activating SK channels (Wolfart and Roeper, 2002). In addition, R-type calcium channels have not been shown to affect neuronal activity in any experiments. However, L-type calcium channels have been shown to play an important role in the regulation of firing activities in DA neurons.

L-type calcium channels are responsible for approximately one third of the total Ca^{2+} currents (Cardozo and Bean, 1995;Durante, et al., 2004;Takada, et al., 2001) and

contribute preferentially to whole-cell Ca^{2+} currents evoked by small depolarizations (Durante, et al., 2004;Xu and Lipscombe, 2001) in DA cells. *In vitro* studies show that L-type calcium channel agonists produce membrane depolarization and subsequently increase firing rates, while antagonists reduce membrane depolarization and decrease firing rates, in DA neurons (Cardozo and Bean, 1995;Chan, et al., 2007;Durante, et al., 2004;Mercuri, et al., 1994;Zhang, et al., 2005). In addition, it has recently been found that the L-type calcium channel antagonist, nifedipine, prevents burst firing and the underlying membrane potential oscillation induced by cholinergic activation in slice preparations (Zhang, et al., 2005).

The role of L-type calcium channels in regulating DA neurons' firing activities parallels their functional importance in addictive and motivational behaviors. For example, repeated amphetamine injections increase the expression of L-type calcium channel subunits in the VTA (Rajadhyaksha, et al., 2004) and repeated stimulation of L-type calcium channels in the VTA mimics the initiation of behavioral sensitization to cocaine (Licata, et al., 2000), while direct injection of L-type calcium channel antagonists into the VTA attenuates the development of psychostimulant-induced behavioral sensitization (Licata, et al., 2004).

L-type calcium channels have unique properties, compared to other voltage-gated Ca^{2+} channels: they are activated by strong depolarizations (high-voltage-activated [HVA]), display a high sensitivity to dihydropyridine (DHP) agonists and antagonists,

have relatively slow activation kinetics, have calcium-dependent inactivation with little voltage-dependent inactivation (long-lasting), and have large single-channel conductance (Lipscombe, et al., 2004). There are four subtypes of L-type calcium channels: $Ca_v1.1$, $Ca_v1.2$ (formerly $\alpha 1C$), $Ca_v1.3$ (formerly $\alpha 1D$) and $Ca_v1.4$ (Lipscombe, et al., 2004). Only $Ca_v1.2$ and $Ca_v1.3$ L-type calcium channels are expressed in DA neurons, especially in the cell body and proximal dendrites (Hell, et al., 1993;Takada, et al., 2001). Functionally, $Ca_v1.3$ L-type calcium channels display atypical properties for L-type calcium channels: having low activation thresholds and showing less sensitivity to DHP antagonists (Durante, et al., 2004;Xu and Lipscombe, 2001). Pharmacologically, there is no effective way to isolate the contribution of $Ca_v1.2$ and $Ca_v1.3$ subtypes. $Ca_v1.3$ and $Ca_v1.2$ transgenic mice, however, provide a means to study the role of L-type calcium channel subtypes (Platzer, et al., 2000). Recently, a dominant role for $Ca_v1.3$, a subtype of the L-type calcium channel that operates at close to resting membrane potentials, was reported in sustain pacemaking activities of DA cells in adult mice(Chan, et al., 2007).

1.4 Rationale and hypotheses for the present thesis

The foregoing overview summarizes the current available information about the functional role and regulation of electrical activities of DA neurons in the VTA, with a special focus on the L-type calcium channels. Up to the present, L-type calcium channels have been shown to play an important role in regulating the firing activity in DA neurons in the midbrain (Cardozo and Bean, 1995;Chan, et al., 2007;Durante, et al., 2004;Mercuri,

et al., 1994;Zhang, et al., 2005), while the signaling pathways preceding and following the L-type calcium channels to mediate this function are still unclear. The work in this thesis is concerned with unraveling the details of the signaling pathway(s) underlying L-type calcium channel regulation of the firing activities of DA neurons.

As previously reported, L-type calcium channels mediate the bursting induced by the general cholinergic activator carbachol (Zhang, et al., 2005). In the first series of experiments (Chapter 2), I hypothesize that L-type calcium channels can directly induce bursting by way of activating calcium-related signaling pathways in DA neurons. In this set of experiments, I asked whether direct activation of L-type calcium channels induces bursting of DA neurons, and if it does, what cellular and synaptic machinery is involved such as activating autoreceptors on the cell body, enhancing neurotransmitter release from afferents, mobilizing internal calcium stores, and activating calcium-activated potassium channels or activating calcium-dependent kinases.

The second series of experiments was derived from the results of the first series that showed calcium influx through L-type calcium channels generates protein kinase M (PKM) to induce bursting of DA neurons. These experiments test the hypothesis that protein kinase C (PKC) might mediate the excitation of carbachol, NMDA or AMPA. In this set of experiments (Chapter 3), I asked whether PKC or L-type calcium channels mediates the firing rate increase induced by application of carbachol, NMDA or AMPA, and if it does, whether they are sequentially coupled in order to modulate firing activity.

In the third series of experiments (Chapter 4), I tested the hypothesis that different subtypes of L-type calcium channels mediate different aspects of the firing activity of DA neurons, which was also derived from the first series of experiments. I asked which subtypes of L-type calcium channels is involved in the spontaneous pacemaker firing and L-channel-induced bursting and whether different subtypes play different roles in the regulation of firing activity by using the $Ca_V1.2$ transgenic mouse strain.

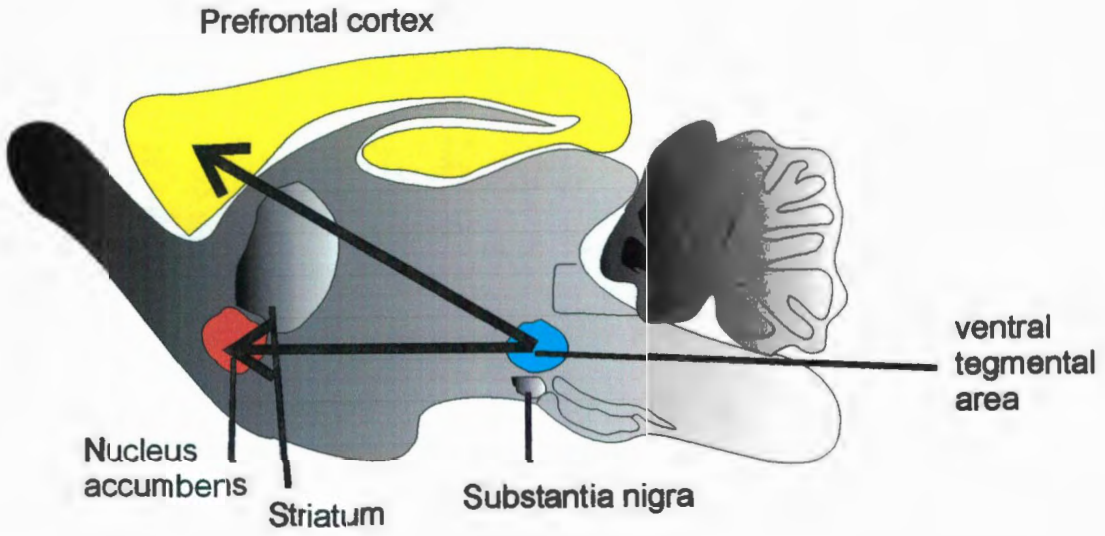

Figure 1.1 The mesolimbocortical system. This simplified diagram shows projections from the ventral tegmental area to the prefrontal cortex and nucleus accumbens (dense lines with arrows).

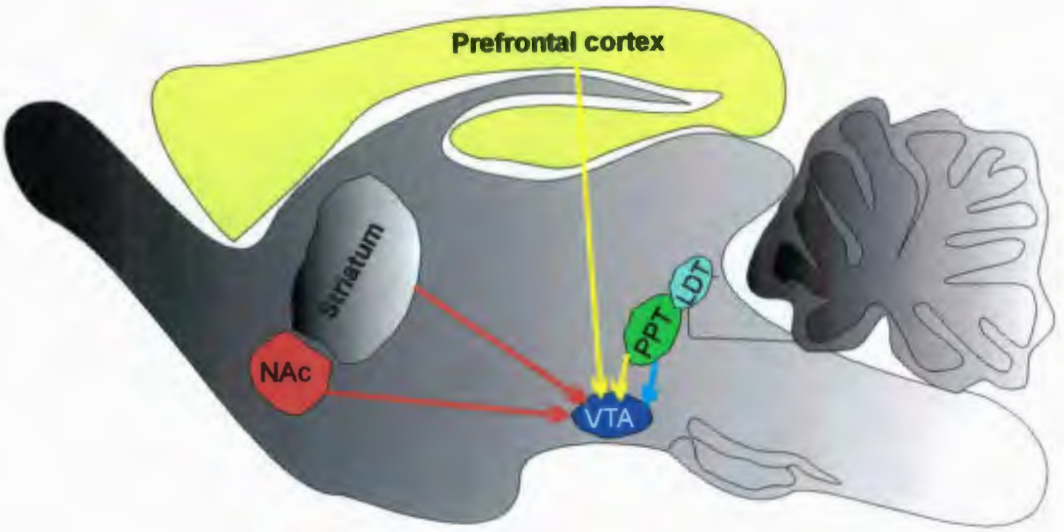

Figure 1.2 The connectivity of the ventral tegmental area (VTA). This simplified diagram shows the afferent inputs (dense lines with arrows) to the VTA. The red lines represent the major GABAergic inputs from the nucleus accumbens (NAc); the yellow lines represent the major glutamatergic inputs from the prefrontal cortex, the pedunculopontine tegmental nuclei (PPT) and the laterodorsal tegmental nuclei (LDT); and the yellow lines represent the major cholinergic inputs from the PPT and the LDT.

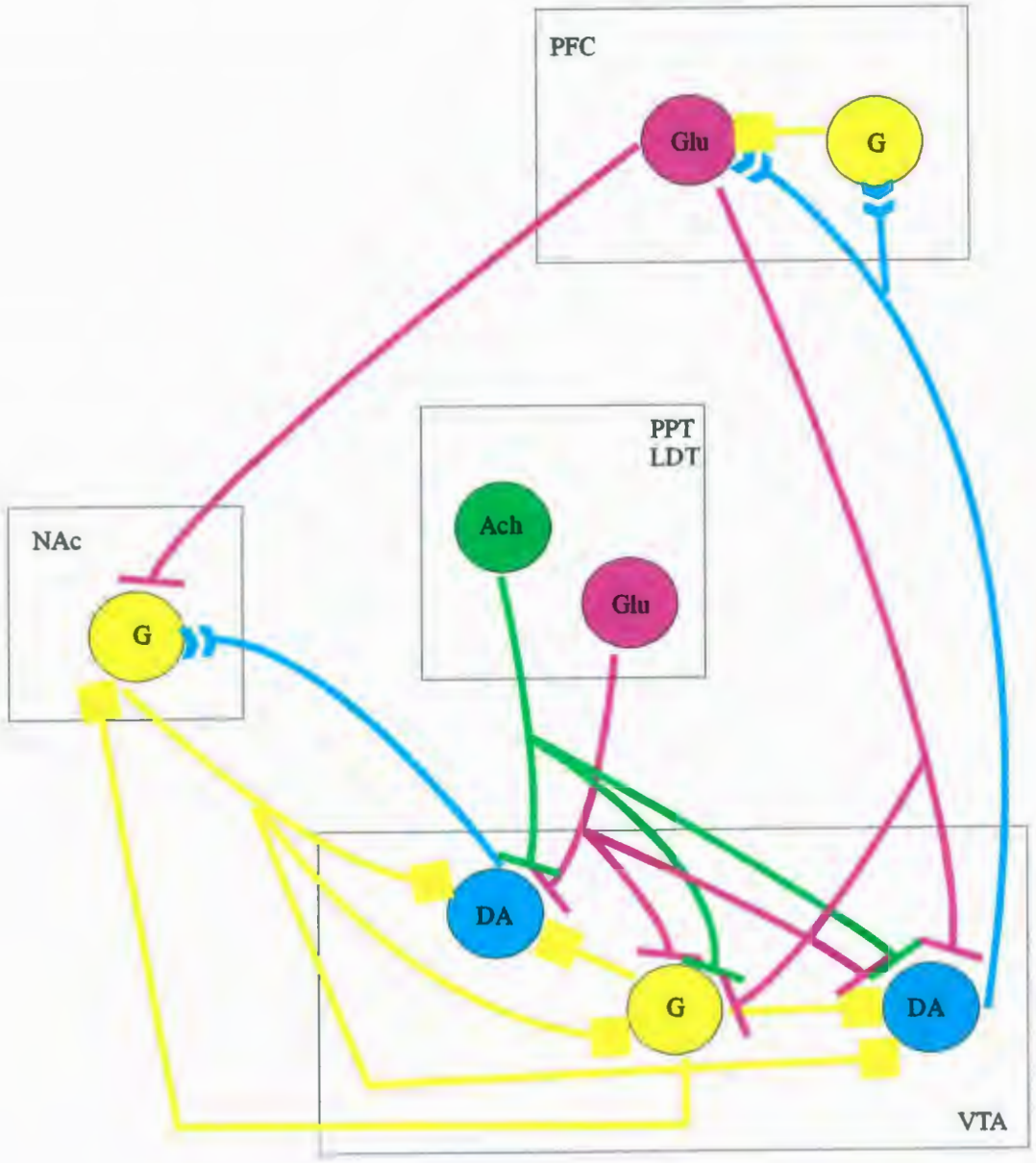

Figure 1.3 A synopsis of the neuronal connections and their interactions with each other in the ventral tegmental area (VTA). DA, DAergic neuron; G, GABAergic neuron; Glu, glutamatergic neuron; ACh, cholinergic neuron; PFC, prefrontal cortex; NAc, nucleus accumbens; PPT, pedunculopontine tegmental nuclei; LDT, laterodorsal tegmental nuclei.

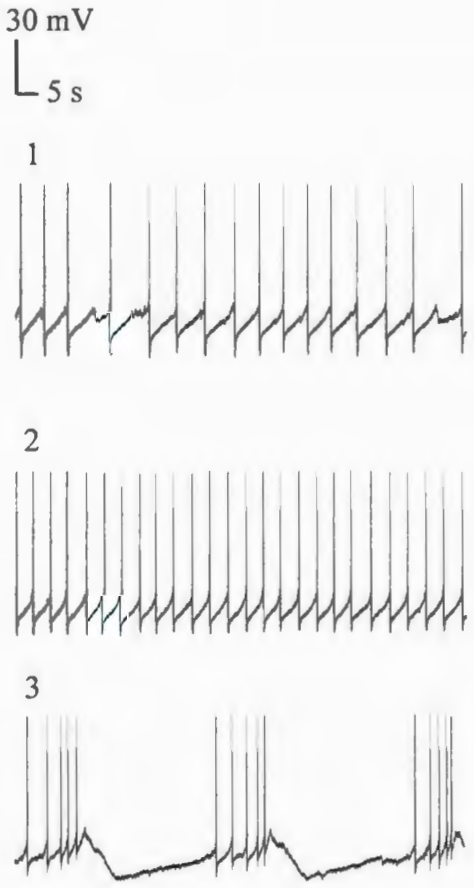

Figure 1.4 Different firing patterns of dopaminergic neurons.

1. irregular firing 2. regular firing 3. burst firing.

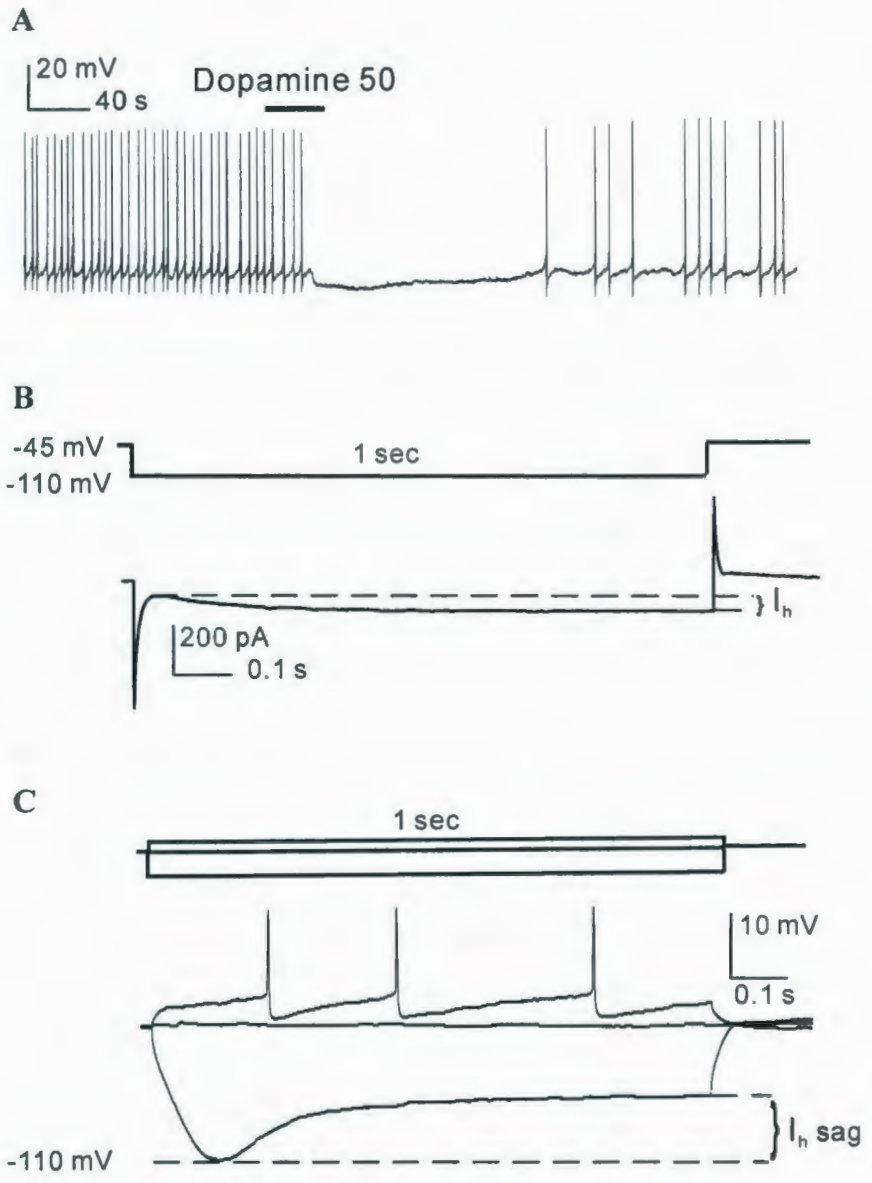

Figure 1.5 Two electrophysiological markers that I used to identify DA neurons. A. Continuous current clamp recording from a representative cell showing the inhibitory effects. **B.** Voltage clamp recording showing a gradual increase in an inward current (I_h current) in response to a voltage step to -110 mV for 1 sec from the holding potential (-45 mV). **C.** Current clamp recording showing a typical Ih "sag" when current injections were adjusted to result in a peak hyperpolarization of about -110 mV.

Co-authorship Statement

I, Yudan Liu, hold a principle author status for all the manuscript chapters (Chapter 2-4) in my thesis. I also have a second-authored paper which is not included in this thesis. However, each manuscript is co-authored by my supervisor and co-workers, whose contributions have greatly facilitated the development of my hypotheses in the manuscripts, the practical aspects of my experiments and the manuscript writing as described below.

Chapter 2, titled "Calcium Influx through L-type Calcium Channels Generates Protein Kinase M to Induce Burst Firing of Dopaminergic Cells in the Rat Ventral Tegmental Area", is co-authored by Drs. Xihua Chen and Jules Dore. As the principle author, I participated in the experimental design and accomplished all the experimental work. I accomplished the first draft writing. Dr. Chen contributed greatly to the design of the research in this paper, and the completion and improvement of the paper. Dr. Dore provided substantial technical training and support in the experiments using Western blotting technique, and the editing of the paper.

Chapter 3, titled "Cholinergic Excitation of Dopaminergic Cells Depends on Sequential Activation of Protein Kinase C and the L-type Calcium Channel in Ventral Tegmental Area Slices" is co-authored by Dr. Xihua Chen. As the principle author, I participated in the development of the experimental designs and performed all the

experimental work and finished the first draft of the paper. Dr. Xihua Chen contributed greatly to the design of the research and the completion and improvement of the paper.

Chapter 4, titled "$Ca_v1.3$ L-type Calcium Channels Mediate Single Spike and Burst Firing of VTA DA Neurons in Mice" is co-authored by Dr. Xihua Chen, Dr. Jules Dore and Meghan Harding. As the principle author, I participated in all of the electrophysiological experiments and most of the PCR experiments. I accomplished the first draft writing. Dr. Xihua Chen contributed greatly to the design of the research and the completion and improvement of the manuscript. Dr. Jules Dore provided substantial technical training and support in genotyping DHP knock-in mice using the PCR technique. Meghan Harding co-worked with me on PCR experiments for breeding and genotyping the mice.

Chapter 2. Calcium Influx through L-type Calcium Channels Generates Protein Kinase M to Induce Burst Firing of Dopaminergic Cells in the Rat Ventral Tegmental Area

(published in J Biol Chem 282: 8594-603, 2007)

2.1 Introduction

DA projections from the VTA constitute the mesolimbocortical system that underlies drug abuse and schizophrenia, primarily as the result of increased DA transmission (Kelley, 2004;Koob, 2000;Seamans and Yang, 2004). The strength of DA transmission is regulated by the interplay between spike-dependent DA release, DA reuptake and autoreceptor-mediated negative feedback. Essentially, more intense spiking releases more DA at the terminal, which in turn activates the negative feedback mechanism to shut the system off. For enduring DA transmission at greater intensity, it would require a higher accumulation of DA at the synapse without triggering the negative feedback machinery. Burst firing is one such mode of discharge that has been shown to enhance DA transmission (Floresco, et al., 2003;Garris, et al., 1994;Gonon, 1988;Suaud-Chagny, et al., 1992) by saturating reuptake transporters and reducing autoreceptor inhibition (Chergui, et al., 1994).

DA cells in the VTA are capable of both pacemaker-like and burst firing (Hyland, et al., 2002), the latter being shown to signal novelty and salient stimuli in whole animal

studies (Tobler, et al., 2005). DA cells in slices predominantly display pacemaker-like firing, burst firing can be induced by surrogate synaptic stimulation with bath application of glutamatergic or cholinergic agonists (Johnson, et al., 1992;Zhang, et al., 2005) that mobilize Ca^{2+}. Ca^{2+} entry has been shown to be pivotal in regulating firing patterns of DA neurons. Intracellular administration of Ca^{2+} evokes burst firing while intracellular Ca^{2+} chelators block it (Grace and Bunney, 1984a). Our previous study shows that carbachol, a general cholinergic agonist, induces burst firing primarily by promoting Ca^{2+} influx through L-type calcium channels (Zhang, et al., 2005). These channels are responsible for approximately one third of total Ca^{2+} currents of DA neurons (Cardozo and Bean, 1995;Durante, et al., 2004;Takada, et al., 2001) and contribute preferentially to whole-cell Ca^{2+} currents evoked by small depolarizations (Durante, et al., 2004;Xu and Lipscombe, 2001). In line with this, L-type calcium channels have been shown to be involved in spontaneous and burst firing (Johnson and Wu, 2004;Mercuri, et al., 1994;Nedergaard, et al., 1993;Zhang, et al., 2005).

Activation of L-type calcium channels has been shown to modulate synaptic strength in DA cells (Bonci, et al., 1998). Additionally, L-type calcium channels gate Ca^{2+}-release from internal Ca^{2+} stores, activate plasma membrane Ca^{2+}-dependent K^+ channels, as well as several Ca^{2+}-dependent kinases such as PKC, calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase A (PKA) (Berridge, et al., 2003). These kinases are capable of phosphorylating a variety of ion channels to regulate the excitability of neurons (Levitan, 2006). Finding the mechanism that controls the firing mode of DA cells

would provide a vital means of modulating the system in both normal and disease conditions. Here, I present results that Ca^{2+} influx through L-type calcium channels activates a Ca^{2+}-dependent protease which in turn cleaves PKC to generate a labile fragment that is constitutively active (termed protein kinase M, PKM) to induce burst firing in DA neurons.

2.2 Methods

All procedures involving animal handling and tissue harvesting were in accordance with guidelines set by the Institutional Animal Care Committee at the Memorial University of Newfoundland. Animals were housed in a temperature-controlled facility under a 12 hr/12 hr light/dark cycle with food and water available *ad libitum*.

2.2.1 Slice preparation

Sprague-Dawley rat pups (9-21 days old) of either sex were deeply anaesthetized with halothane and killed by chest compression. The skull was quickly opened to expose the brain, which was cooled *in situ* with ice-cold, carbogenated artificial cerebrospinal fluid (ACSF, composition: 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 18 mM $NaHCO_3$, and 11 mM glucose, pH 7.4 when bubbled with 95% O_2 and 5% CO_2). The brain was removed and a block containing the midbrain was cut horizontally on a Leica vibratome (VT 1000, Heidelberger, Germany). Tissue slices

(400 μm thick) were allowed to recover at room temperature (22°C) in carbogenated ACSF for at least 1 h prior to recording. Slices were further trimmed to fit into a recording chamber and continuously perfused with carbogenated ACSF at a rate of 2-3 ml min^{-1} at room temperature. For PKC depletion experiments, one of the two VTA slices from the same animal was incubated in ACSF with or without 1-2 μM phorbol 12-myristate 13-acetate (PMA) for 20-24 h at room temperature in a partially sealed beaker continuously bubbled with carbogen.

2.2.2 Patch clamp recording

All recordings were made from the VTA identified under a dissecting microscope (Leica MZ6). Patch electrodes were prepared from KG-33 glass micropipettes (OD 1.5 mm, Garner Glass CO., Claremont, CA, USA) on a P-97 Brown-Flaming micropipette puller (Sutter Instruments, Novato, CA, USA). For nystatin-perforated patch clamp recording, glass electrodes were filled to the tip with intracellular solution (120 mM potassium acetate, 40 mM HEPES, 5 mM $MgCl_2$, and 10 mM EGTA with pH adjusted to 7.35 using 0.1 N KOH) and then back-filled with the same solution containing 450 μg ml^{-1} nystatin and Pluronic F127, yielding a tip resistance of 4-8 MΩ. For conventional whole-cell recording, electrodes were filled with a solution containing 126 mM potassium gluconate, 10 mM KCl, 0.2 mM EGTA, 10 mM HEPES, 4 mM MgATP and 0.3 mM Na_3GTP with pH adjusted to 7.35 and osmolarity to 295 mOsm, yielding a resistance of 2-4 MΩ. Gigaohm seals were made using a Warner PC-505B (Warner Instruments Inc.,

Hamden, CT, USA) or a MultiClamp 700B (Axon Instruments, Foster City, CA, USA) amplifier. Signals were sampled at 5 kHz and digitized by DigiData 1320A using pCLAMP (versions 8 and 9) software (Axon Instruments).

Selection of nystatin-perforated cell recordings in current clamp mode was determined by the size of the action potential, since many VTA cells were spontaneously active. After adequate partitioning of nystatin into the membrane, action potentials overshot 0 mV and measured at least 50 mV. Quality of conventional whole-cell recordings was assessed by a brief voltage step (-20 mV, 10 ms) from the holding potential (-55 mV). Only cells that had an access resistance of <30 MΩ and an input resistance of >200 MΩ were included. Cells whose access resistance increased significantly during the course of recording (>20%) were discarded. Episodic protocols were used to induce I_h and derive passive characteristics of the cell such as current-voltage relationship and input resistance. Current pulses for I_h induction were 1 s and the intervals between pulses were 8 s to allow complete recovery of I_h channels. Cells that displayed a prominent I_h and an apparent DA-induced hyperpolarization were identified as putative DA cells (Figure 1.5) (Johnson, et al., 1992;Lacey, et al., 1989;Zhang, et al., 2005).

Components of extracellular and intracellular solutions were purchased from bulk distributors Fisher Scientific (Nepean, Ontario, Canada) and VWR International (Missisauga, Ontario, Canada). All other chemicals were obtained from Sigma (St Louis,

MO, USA) and Tocris (Ellisville, MO, USA). Chemicals were dissolved in deionized water or DMSO (0.1% final concentration) as required. Aliquots of stock solutions were kept at –30°C. Prior to application, an aliquot was diluted to working concentration and applied to the ACSF bath. PKM (Sigma) was kept at –80 °C and was diluted to 1 unit ml^{-1} immediately before use in the internal solution for conventional whole-cell recording. DA solution was made fresh daily with an equimolar concentration of the antioxidant disodium metabisulfite.

2.2.3 Western blotting

Phosphorylated PKC and PKM were detected by a phospho-PKC (pan) antibody (Cell Signaling, CA, USA) that recognizes PKCα, βI, βII, δ, ε, η and θ isoforms only when phosphorylated at a carboxyl-terminal residue homologous to Ser660 of PKCβII. Ser660 is in the catalytic domain of PKC and allows simultaneous visualization of phosphorylated PKC and PKM. VTA slices from the same animal were hemisected to give equivalent halves for control and experimental groups. Slices were trimmed to contain the VTA and part of the substantia nigra and were homogenized in RIPA [1x PBS, 1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 0.5% deoxycholate, 50 mM β-glycerophosphate, 50 mM sodium fluoride, 5 mM EDTA, 0.1% sodium orthovanadate, 0.1% SDS, 75 ng/ml phenylmethylsulfanyl fluoride (PMSF) and 1x complete protease inhibitor (Roche Diagnostics, QC, Canada)]. Insoluble materials were removed by centrifugation (15,000xg for 10 min), protein concentration for each sample was

determined by BCA™ assay using BSA standard (Pierce, Rockford, USA). Equivalent mass of total protein from slice homogenates were separated on an 8.5% acrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane. The blots were blocked with Blotto (5% w/v non-fat milk in TBST; 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween20) for 1 h at room temperature, then incubated with phospho-PKC (pan) antibody (1: 1,000) diluted in Blotto, rocking overnight at 4°C. Blots were washed 5 times in TBST and followed by goat anti-rabbit HRP-conjugated IgG diluted in Blotto (Cell Signaling, 1: 15,000) for 90 min at room temperature. After five washes in TBST, blots were incubated in enhanced SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, USA) to visualize specific immune complexes using hyperfilm X-ray film (Amersham Biosci., NJ, USA). Blots were then stripped in a solution of 2% SDS, 63 mM Tris-HCl (pH 6.8) and 0.1 M β-mercaptoethanol for 30 min at 50°C. Stripping solution was removed by two washes with TBST at room temperature, followed by blocking with Blotto and incubation with anti-β-actin antibody (Sigma, 1: 4,000) to act as loading controls. Quantification was obtained from densitometric measurements of immunoreactive bands using Gel Logic100 imaging system with Gel Logic200 Software (Kodak, USA).

2.2.4 Data analysis

Electrophysiological data were analyzed offline with Mini Analysis (Synaptosoft Inc., Decatur, GA, USA) and pCLAMP software. Basal firing frequencies were averaged

values of at least 5 min stable baseline recording. I_h was measured as the difference in current or voltage between instantaneous and steady-state readings. Analysis of firing behavior was based on interspike intervals (ISIs) measured with the Mini Analysis program. Averaged as well as instantaneous firing frequencies were derived from those intervals. Coefficient of variance (CV) was calculated as the mean of ISIs over a 1-min period divided by their standard deviation. To compare CV values between cell groups, they were normalized against the mean CV value of the first 5 min. Relative density of ISIs in 2-second bins were plotted to reveal the distribution of a given ISI series, and the resulting histogram was fitted to a Lowess function. Burst firing was defined as two spikes or more in each bursting cycle at a frequency higher than non-bursting periods and separated by a post-burst hyperpolarization, which was visually judged and quantified. For western blotting analysis, density of phospho-PKM and PKC bands were normalized against the density of β-actin from the same sample.

Data were expressed as means and standard errors of the mean (SEM). Statistical comparisons of electrophysiological data were performed using two-tailed unpaired Student's t test. Western blotting data were compared using paired t tests since each control and experimental pair contained hemisected slices from the same animal. Values were considered significant when $P < 0.05$.

2.3 Results

Except when indicated, experiments were done using the nystatin-perforated whole-cell recording method at room temperature. Dialysis of PKM through the electrode into recorded cells was done using conventional whole-cell recording. Only DA cells in the VTA identified according to criteria outlined in Methods section were included. In nystatin-perforated recording, the average hyperpolarization following a brief application of 50 μM DA (within 90 sec) was –8.29±0.57 mV (n=71, excluding cells used for PKC depletion test). Most cells (47 of 71, 66%) were spontaneously active with single spike firing at a low basal firing frequency of 0.41±0.04 Hz; the remainder (24 of 71, 34%) were quiescent during baseline recordings.

2.3.1 Opening of L-type calcium channels converts firing patterns

Bath application of 1-4 μM FPL 64176, a benzoylpyrole site L-type calcium channel opener, for 5-10 min converted firing patterns from quiescent state or single spiking to burst firing in 80.3% of treated cells (57 of 71), application of 1 μM FPL 64176 induced burst firing in 63.5% cells (33 of 52). The responses were spike-dependent. Percentage of converted burst firing in spontaneously firing cells (41 of 47, 87.2%) was much higher than that in quiescent cells (16 of 24, 66.7%), a response that appeared to be related to their different resting membrane potentials (–49.35±0.54 mV for spiking cells vs. –52.52±0.92 mV for quiescent cells, $P<0.05$). The responses were dose-dependent: 2 μM

FPL 64176 induced burst firing in 9 of 17 cells that did not respond to 1 μM and similarly, 4 μM induced burst firing in 4 of 10 cells that did not respond following a dose of 2 μM.

In cells that were spontaneously firing, FPL 64176 first induced a membrane depolarization (2.00±0.38 mV, n=41) accompanied by a 48.8±9.3% increase in firing rate in the first 5 minutes of drug application, burst firing started after a varying period of latency. The lag between the start of drug application and burst firing ranged 5-25 min with an average of 13 min (Figure 2.1A, 2.1F). Within a burst firing cycle, action potentials were fired with increasing frequencies followed by a pronounced post-burst hyperpolarization (Figure 2.1B). The average intra-burst firing frequency (1.12±0.13 Hz, n=41) was much higher than basal tonic frequency (0.43±0.05 Hz). The development of burst firing in quiescent cells (n=16) was slightly different: they responded to FPL 64176 with a membrane depolarization (1.38±0.38 mV) followed by a sudden appearance of burst firing (Figure 2.1D) or irregular single spiking that evolved into burst firing (Figure 2.1E). The L-type calcium channel-mediated burst firing was long lasting even after prolonged washout up to 3 hours, however, it was readily inhibited by the L-type calcium channel blocker nifedipine at a range of doses (1-10 μM, n=29). Density plots of ISIs in 2-second bins showed an ISI distribution that fits to a single Gaussian distribution under control conditions and to a mixed Gaussian distribution following FPL 64176 application (Figure 2.1C). The leftward shift of the main peak indicates higher firing frequencies and

the second peak at much lower frequency represents the long pauses of firing between adjacent bursts.

Bath application of 5 μM (S)-(-)-Bay K8644, a DHP site L-type calcium channel activator, also induced burst firing in 3 of 5 cells tested (Figure 2.1G). The time course of the response was similar to that following FPL 64176, a depolarization and an increase in firing rates followed by a conversion of firing patterns 10-25 minutes after the start of application.

2.3.2 FPL 64176 induces burst firing independent of an intermediate transmitter

L-type calcium channels have been shown to enhance a slow NMDA current in DA neurons (Bonci, et al., 1998), suggesting that L-type calcium channel opening could induce burst firing by promoting glutamate transmission. Because FPL 64176-induced burst firing was long-lasting, I examined the involvement of synaptic mechanisms in two ways: whether induced burst firing persisted in the presence of a cocktail containing 100 μM APV, 10 μM CNQX and 100 μM picrotoxin (blocking NMDA, AMPA and $GABA_A$ receptors, respectively) and whether pretreatment with this cocktail altered the ability of FPL 64176 to induce burst firing. FPL 64176-induced burst firing was still robust following the application of the cocktail for 10-30 min (n=7, Figure 2.2A). Similarly, cells that were treated with the cocktail for 5-10 minutes prior to FPL 64176 (1 μM) application in the presence of the cocktail still responded with burst firing (n=3).

These results suggest that L-type calcium channel opening does not induce burst firing either directly through increased glutamatergic transmission or indirectly by way of GABAergic interneurons in the VTA.

Activation of L-type calcium channels may release DA from the soma and dendrites since this release has been shown to be Ca^{2+}-dependent (Beckstead, et al., 2004;Chen and Rice, 2001). DA acting at somatodendritic autoreceptors forms the short-loop negative feedback to regulate the excitability of DA cells. I therefore examined whether D_2 receptors were involved in burst firing. The D_2 receptor antagonist sulpiride (1-10 μM), which itself did not change the firing activity, applied for 10-15 min to 5 burst firing cells induced by FPL 64176 had no effect on firing patterns (Figure 2.2B). Two cells to which 10 μM sulpiride was applied for 8 min prior to FPL 64176 application in the presence of sulpiride displayed burst firing similar to that induced by FPL 64176 alone. These data suggest that L-type calcium channel activation does not result in somatodendritic DA release to induce burst firing.

2.3.3 Internal Ca^{2+} stores are not involved

Ca^{2+} entry through L-type calcium channels gates ryanodine receptors on internal Ca^{2+} stores to mediate Ca^{2+}-induced Ca^{2+} release (Berridge, et al., 2003). So I tested whether this was involved in burst firing. Cyclopiazonic acid (CPA) and thapsigargin were used to inhibit the sarco-endoreticulum Ca^{2+}-dependent ATPase (SERCA) that

refills internal Ca^{2+} stores. After burst firing was induced by FPL 64176, CPA (20-30 μM) applied for 20-60 min (n=4) or thapsigargin (1-2 μM) applied for 50-70 min (n=3) did not stop the induced burst firing (Figure 2.2C). In three cells that were pretreated with CPA (20 μM) for 30 min, FPL 64176 (1 μM) was still able to induce strong burst firing. These results indicate that internal Ca^{2+} stores are not necessary for L-type calcium channel-mediated burst firing.

2.3.4 Ca^{2+}-dependent protein kinase mediates burst firing

Increased intracellular Ca^{2+} activates Ca^{2+}-sensitive protein kinases such as PKC, PKA and CaMKII that can phosphorylate ion channels including L-type calcium channels themselves and regulate the excitability of neurons (Dzhura, et al., 2000;Lee, et al., 2006;Yang, et al., 2005;Young and Yang, 2004). After burst firing was induced by FPL 64176, bath application of the PKA inhibitor H-89 (5 or 10 μM) for 40-130 min (n=4), the CaMKII inhibitor KN-93 (1-10 μM) for 25-100 min (n=6), or both for 60-70 min (n=3) did not block burst firing (Figure 2.3A). The substrate site PKC inhibitor, chelerythrine (40 μM), applied for 15-44 min (n=6) completely blocked FPL 64176-induced burst firing and the accompanying membrane potential oscillation. This blockade was reversible since burst firing reappeared after washing out chelerythrine for 18-33 min (Figure 2.3B). To establish the role of PKC in burst firing, I further used an inhibitor that binds to the diacylglycerol (DAG) site of the regulatory domain. Calphostin C (1-2 μM) applied for 40-180 min (n=4) was largely ineffective except in one cell where

burst firing slowed marginally but persisted (Figure 2.3C). These results suggest that the catalytic subunit of PKC itself or a kinase with a similar substrate site is involved in burst firing.

2.3.5 Proteolytic cleavage of PKC mediates burst firing

To solve the apparent inconsistency between the two PKC inhibitors, I considered other modes of PKC activation. Ca^{2+} entry has been shown to activate the protease calpain that proteolytically cleaves PKC to form PKM which has been shown to be constitutively active with a very short half-life within the cell (Al and Cohen, 1993;Cressman, et al., 1995;Kishimoto, et al., 1983;Shea, et al., 1994). I therefore tested whether proteolytic cleavage of PKC could explain the inconsistency in response to the two PKC inhibitors. Cells were induced to burst fire by FPL 64176, the calpain inhibitor MDL 28170 (200 μM) was then applied. In all cells tested as such, burst firing reverted to single spike firing in 25-40 min and resumed after washing 20-30 min (n=5, Figure 2.4A). Prior application of MDL 28170 (200 μM) for 30-40 min completely prevented FPL 64176-induced (2-4 μM) burst firing (n=3).

To further validate the role of PKM in L-type calcium channel-induced burst firing, I compared the levels of phospho-PKC and PKM by Western blotting total protein lysates from slices that were treated with (S)-(-)-Bay K8644 or FPL 64176 alone and with MDL 28170 followed by FPL 64176. As shown in Figure 2.4B, phospho-PKC antibody raised

against the catalytic domain visualized three bands: two at approximately 82 kDa corresponding to intact PKC isoforms and another at 45 kDa corresponding to the PKC catalytic unit or PKM. Application of L-type calcium channel opener FPL 64176 (2 μM) or (S)-(-)-Bay K8644 (5 μM) for 30 min caused a considerable increase in phospho-PKM expression (Figure 2.4C). Densitometry of the bands corresponding to phospho-PKM showed that (S)-(-)-Bay K 8644 (224±61% relative to untreated values, n=8, $P<0.05$) or FPL 64176 (244±51% relative to untreated values, n=6, $P<0.05$) significantly increased phospho-PKM levels (Figure 2.4D). The increase by FPL 64176 could be completely blocked by prior treatment with the calpain inhibitor MDL 28170 (200 μM for 30 min; 8±1% of FPL 64176 alone, n=5, $P<0.0001$; Figure 2.4C, D). There were no detectable changes in full-length phospho-PKC levels following (S)-(-)-Bay K8644 (102±10% of control values, n=8, $P>0.05$), FPL 64176 (104±6% of control values, n=6, $P>0.05$) or MDL 28170 with FPL 64176 (95±2% of FPL 64176 alone, n=5, $P>0.05$). These data indicate that PKM levels parallel burst firing behavior.

2.3.6 Burst firing can not be induced after PKC depletion

Since PKM is generated by cleavage of PKC (Cressman, et al., 1995;Shea, et al., 1994), it is an obvious test to determine if burst firing can be induced following PKC depletion. Persistent activation by phorbol esters such as PMA, leads to degradation of PKC isoforms that have the diacylglycerol site and is used experimentally to produce a PKC-depleted cell (McArdle and Conn, 1989;Szallasi, et al., 1994). Slices were incubated

with or without PMA (1-2 μM) for 20-24 hours followed by FPL 64176 (4 μM) to induce burst firing. Regardless of incubation conditions, cells from these slices were no longer spontaneously active although they were capable of firing action potentials (Figure 2.5A). There was no difference in resting membrane potential (control: -50.05±1.36 mV, n=6; PMA-treated: -48.25±1.38 mV, n=7, P>0.05) or input resistance (control: 403.24±56.12 MΩ, n=6; PMA-treated: 293.58±42.93 MΩ, n=7, P>0.05) between control and PMA-treated groups. PMA-treated cells had a significantly smaller DA-induced hyperpolarization (control: 12.03±3.43 mV; PMA-treated: 2.99±0.67 mV, P<0.05). Western blots showed a disappearance in PMA-treated slices of a band around 82 kDa and the concomitant decrease of the PKM band at 45 kDa following FPL 64176 (Figure 2.5B).

There was a clear difference in FPL 64176-induced firing between the two groups. Of 6 cells from control slices, FPL 64176 (4 μM) induced burst firing in 3 (Figure 2.5C1), large membrane potential oscillations in 2 and no change in one cell. Both burst firing and membrane potential oscillation could be blocked by nifedipine (5 μM). Of 7 cells from PMA-treated slices, FPL 64176 (4 μM) only induced an average membrane depolarization of 0.62±0.26 mV with no burst firing or membrane potential oscillation in any of the cells tested (Figure 2.5C2). These data show depletion of PKC decreased the PKM expression and blocked the bursting production, further indicating that PKM levels parallel bursting firing behavior.

2.3.7 Direct loading of PKM induces burst firing

If PKM links L-type calcium channels and burst firing, it would be expected that direct loading of PKM (1 unit/ml pipette solution) into the cells through the conventional whole-cell recording pipette should induce similar firing mode switching. Out of seven cells, three had single spike firing initially that became irregular and clustered by 3-5 min, with burst firing appearing at 9-12 min. Burst firing was strong and stable for 20-30 min (Figure 2.6A) and was qualitatively similar to burst firing induced by FPL 64176. When burst firing was prevalent, density plots of ISIs showed a typical two-peak ISI distribution (Figure 2.6C2). In another 2 cells, single spike firing became burst-like characterized by clustered spikes followed by a pause without a post-burst hyperpolarization. Only in 2 cells was no response seen. The control experiments were done using the same internal pipette solution without PKM and all 6 cells tested maintained regular firing during the 40 min recording period (Figure 2.6B). Plotting normalized coefficient of variance values against the average value of the first 5 min (Figure 2.6D) revealed that PKM significantly increased variance while it remained unchanged in control cells (unpaired t test on areas-under-the-curve in arbitrary units, control: 3323.38±291.91, PKM: 5001.95±427.84, $P<0.05$), indicating that PKM disrupts regular spiking and promotes burst firing.

2.3.8 Activation of NMDA and cholinergic receptors increases PKM expression

To test whether PKM is involved in the physiological regulation of burst firing in DA neurons, glutamate or cholinergic agonists known to induce burst firing in DA cells were used to stimulate VTA slices and the amount of PKM was detected by Western blotting. Hemisected slices from the same animal were treated with or without NMDA (20 μM) or carbachol (20 μM) for 20 min to test whether it increased the levels of phosphorylated PKM. As shown in Figure 2.7A, application of either the glutamate agonist NMDA or cholinergic agonist carbachol caused a considerable increase in phospho-PKM expression. Densitometry of the bands corresponding to phospho-PKM of six independent experiments (Figure 2.7B) showed that both NMDA (159±26% relative to untreated values, $P<0.05$) and carbachol (154±41% relative to untreated values, $P<0.05$) significantly increased phospho-PKM levels. Since glutamate and cholinergic inputs are major synaptic modulators of DA cells, these data indicate that PKM is involved in the synaptic regulation of firing behavior of DA cells.

2.4 Discussion

Burst firing is an important property of DA cells in the VTA to signal novelty and salience that are associated with normal or abnormal expression of motivation and reward. I have previously reported that the cholinergic agonist carbachol induces a calcium-dependent burst firing of DA cells by promoting Ca^{2+} influx through L-type

calcium channels. This paper studies how L-type calcium channel activation induces burst firing. In this study, I observed that direct activation of L-type calcium channels converted firing patterns of VTA DA cells independent of glutamate, dendritically released DA or Ca^{2+} released from internal stores. Nor did Ca^{2+} influx through L-type calcium channels activate PKA or CaMKII to induce burst firing. I have shown that Ca^{2+} influx through L-type calcium channels activates calpain proteases that in turn cleave PKC, releasing the short lived, active PKM fragment to produce burst firing. Disruption at any point along this chain of events results in an inability to cause burst firing of DA neurons in the VTA. I have also shown that PKM is involved in the regulation of DA cells under physiological stimulations where the NMDA type of glutamate receptors or cholinergic receptors were activated.

2.4.1 L-type calcium channel-induced burst firing does not require an intermediate transmitter

L-type calcium channels are primarily expressed in the soma and dendrites of DA cells in the VTA area (Takada, et al., 2001) although they have been shown to modulate synaptic transmission to the VTA (Bonci, et al., 1998). Glutamate acting at the NMDA receptors mediates a slow excitatory synaptic transmission to VTA DA cells (Mercuri, et al., 1996) that can be enhanced by L-type calcium channel activation (Bonci, et al., 1998), suggesting that the effects of L-type calcium channel activation could be secondary to increased glutamate transmission. This is particularly relevant since NMDA is one of the

pharmacological tools used to induce burst firing in DA neurons (Johnson, et al., 1992;Johnson and Wu, 2004). Our results showing that combined synaptic blockade at the NMDA, AMPA and $GABA_A$ sites before or after L-type calcium channel activation did not alter burst firing suggest that glutamate transmission is not involved in L-type calcium channel-induced burst firing. Alternatively, activation of L-type calcium channels increases firing (Mercuri, et al., 1994) and subsequently releases DA from somatodendritic sites in order to terminate firing by D_2-mediated autoinhibition (Beckstead, et al., 2004). Autoinhibition wanes as DA is taken back by DAT and a new cycle starts. This mode of action appears logical since all the proposed mechanisms have been shown to work in DA cells. However, L-type calcium channel activation induced strong burst firing that was not blocked by the D_2 receptor antagonist sulpiride. Blocking the D_2 receptor before application of L-type calcium channel opener did not alter burst firing either, arguing against a role for somatodendritic D_2 receptors in L-type calcium channel-induced burst firing.

It has been shown previously that burst firing of DA cells depends on Ca^{2+} oscillations (Zhang, et al., 2005), a phenomenon that could be supported by so-called Ca^{2+}-induced Ca^{2+} release from internal stores. The L-type calcium channel has been shown to directly gate Ca^{2+} releasing channels on the membrane of sarcoplasmic reticulum (Berridge, et al., 2003). The IP_3 channels have a bell-shape sensitivity relationship with intracellular Ca^{2+} levels (Berridge, et al., 2003) so that their conductance increases initially with higher Ca^{2+} concentrations, further increases in Ca^{2+} reduces their

conductance capabilities. Also, Ca^{2+} release from internal stores has been found to mediate a slow inhibition in DA cells taking hundreds of milliseconds to develop (Fiorillo and Williams, 1998). This is on a similar time scale to burst firing cycles I observed in slice preparations. These mechanisms can possibly produce a biphasic response that fits the membrane oscillation I observed. In my experiments, burst firing induced by the L-type calcium channel opener FPL 64176 persisted when internal stores were depleted by prolonged inhibition of the refilling enzyme SERCA, suggesting that internal stores do not play an obligatory role in burst firing.

2.4.2 Ca^{2+} influx generates PKM to induce burst firing

The opening of L-type calcium channels did not induce burst firing within the first 5 minutes following FPL 64176 application although Ca^{2+} influx had already changed the excitability of the cells in the form of membrane depolarization and increased firing frequency. Burst firing emerged 5-25 min following the start of drug application and lasted for hours. The lag seems to indicate that Ca^{2+} entry activated a signaling cascade that takes time to develop or a second messenger must migrate between the intracellular compartments. Since protein kinase activation requires multiple stages of phosphorylation and translocation (Liu and Heckman, 1998), the involvement of Ca^{2+}-dependent protein kinases presents a reasonable mechanism underlying Ca^{2+}-dependent burst firing. For example, CaMKII has been shown to become persistently active during Ca^{2+} oscillations to enhance L-type calcium channels (Hudmon, et al., 2005). Similarly, PKA has long

been associated with adrenergic enhancement of L-type calcium channel activity (Hoogland and Saggau, 2004;van der Heyden, et al., 2005). The involvement of these kinases could explain why L-type calcium channels continue operating despite high intracellular Ca^{2+} levels. Channel phosphorylation has been suggested to reduce the Ca^{2+}-dependent inactivation of L-type calcium channels (Budde, et al., 2002). The fact that burst firing persisted in the presence PKA and CaMKII blockers supports the concept that they are not necessary for Ca^{2+}-dependent burst firing.

The striking result that chelerythrine, a PKC catalytic domain inhibitor, reversibly blocked burst firing, whereas calphostin C, a regulatory domain inhibitor did not, raised the possibility that Ca^{2+} influx through L-type calcium channels activates a PKC-like kinase or PKC operates in an atypical fashion. Elevated intracellular Ca^{2+} has been shown to activate calpain (Goll, et al., 2003), a protease that cleaves the PKC catalytic domain from the regulatory domain (Al and Cohen, 1993;Cressman, et al., 1995;Kishimoto, et al., 1983) to generate PKM. My results support this mode of PKC action and its regulation of burst firing through: 1) L-type calcium channel openers induced burst firing accompanied by increased levels of phosphorylated PKM; 2) inhibition of the Ca^{2+}-dependent protease calpain reduced the level of phosphorylated PKM and concomitantly blocked burst firing; 3) PKM is generally thought to be a proteolytic product of PKC (Al and Cohen, 1993;Cressman, et al., 1995;Kishimoto, et al., 1983) and depletion of PKC isoforms following prolonged incubation with PMA prevented L-type calcium channel-induced burst firing; 4) direct loading of the cell with purified PKM through the recording pipette

induced burst firing in a similar manner to Ca^{2+} influx, with the temporal development of burst firing being consistent with diffusion of PKM into the cell. The evidence collectively indicates that Ca^{2+} influx through the L-type calcium channel activates proteolytic cleavage of PKC to generate PKM that facilitates the development and maintenance of burst firing of DA cells.

2.4.3 PKC is functionally important for DA-related conditions

PKM was found in the rat brain in the 1970s (Inoue, et al., 1977) but its function remained obscure until recent reports associating it with learning and memory (Osten, et al., 1996;Sacktor, et al., 1993). Several PKC isoforms have been found to give rise to proteolytic PKM counterparts, and it is reasonable to assume that all members of the PKC family are capable of generating their respective PKMs because of their similar structure of the hinge region that joins the regulatory and catalytic domains. My results did not identify the parent PKC isoform that was cleaved to form PKM, it may involve several PKC isoforms, since many are expressed in DA neurons (Yoshihara, et al., 1991). The PKM used for cellular loading is derived from enzymatic digestion of total PKC, but from the PKC depletion experiments, it appears that the PKM involved in burst firing is generated by the isoforms with the diacylglycerol site. PKC has been implicated in addiction and motivation. Injection of PKC inhibitors into the VTA reduces cocaine-induced DA release in the NAc (Steketee, 1993) and delays the onset of behavioral sensitization (Steketee, 1994;1997). Repeated administration of cocaine

increases overall PKC activity in the VTA which may initiate behavioral sensitization (Steketee, et al., 1998). Being persistently active, PKM could fulfill all of these behaviors involving PKCs. More importantly, PKM could achieve all this by its role in burst firing reported here as burst firing elevates terminal DA more effectively and all of these behaviors involve increased DA transmission. In addition, addiction is a learned behavior and PKM has been shown to facilitate learning and memory in rats (Osten, et al., 1996;Sacktor, et al., 1993). Taken together, PKC and its cleaved product PKM might play a significant role in central DA transmission and its related pathologies such as drug abuse. It is especially encouraging that activation of the NMDA and cholinergic receptors increased PKM because these receptors not only mediate synaptic regulation of DA cells, but they are also known to induce burst firing in DA cells.

2.4.4 Mechanisms of PKM-induced burst firing

There are many factors contributing to the strength of DA transmission. Besides the efficiency of the release machinery, it is usually agreed that DA reuptake and autoreceptor-mediated inhibition are important regulators of DA transmission. Released DA is rapidly taken back to the terminal by DAT, whereas D_2 autoreceptors at both terminal and somatodendritic sites respond to increased DA levels by a negative feedback loop that inhibits DA cell activity. PKC has been shown to modulate both processes. Activation of PKC leads to a decrease in DAT capacity (Daniels and Amara, 1999;Melikian and Buckley, 1999) due to accelerated internalization (Holton, et al.,

2005;Sorkina, et al., 2005), reduced recycling (Loder and Melikian, 2003) and degradation (Miranda, et al., 2005). Additionally, PKC activation enhances phosphorylation, desensitization and trafficking of D_2 receptors (Namkung and Sibley, 2004) resulting in a dampened negative feedback. In PMA treated slices, somatodendritic autoreceptors' responses to DA were blunted, it remains to be tested whether these counterparts in the terminal are similarly affected. Together the effects of PKC on DAT and autoreceptor inhibitory feedback, with my results that the proteolytic product of PKC (PKM) induces burst firing, a firing mode that is more effective in increasing DA release, it is a tantalizing possibility that PKC serves as a signaling hub, integrating different aspects of DA transmission to boost its functionality over a long period of time. This postulate further supports the findings that implicate PKC in addiction, a chronic and debilitating condition that has long been thought to be due to enhanced DA transmission.

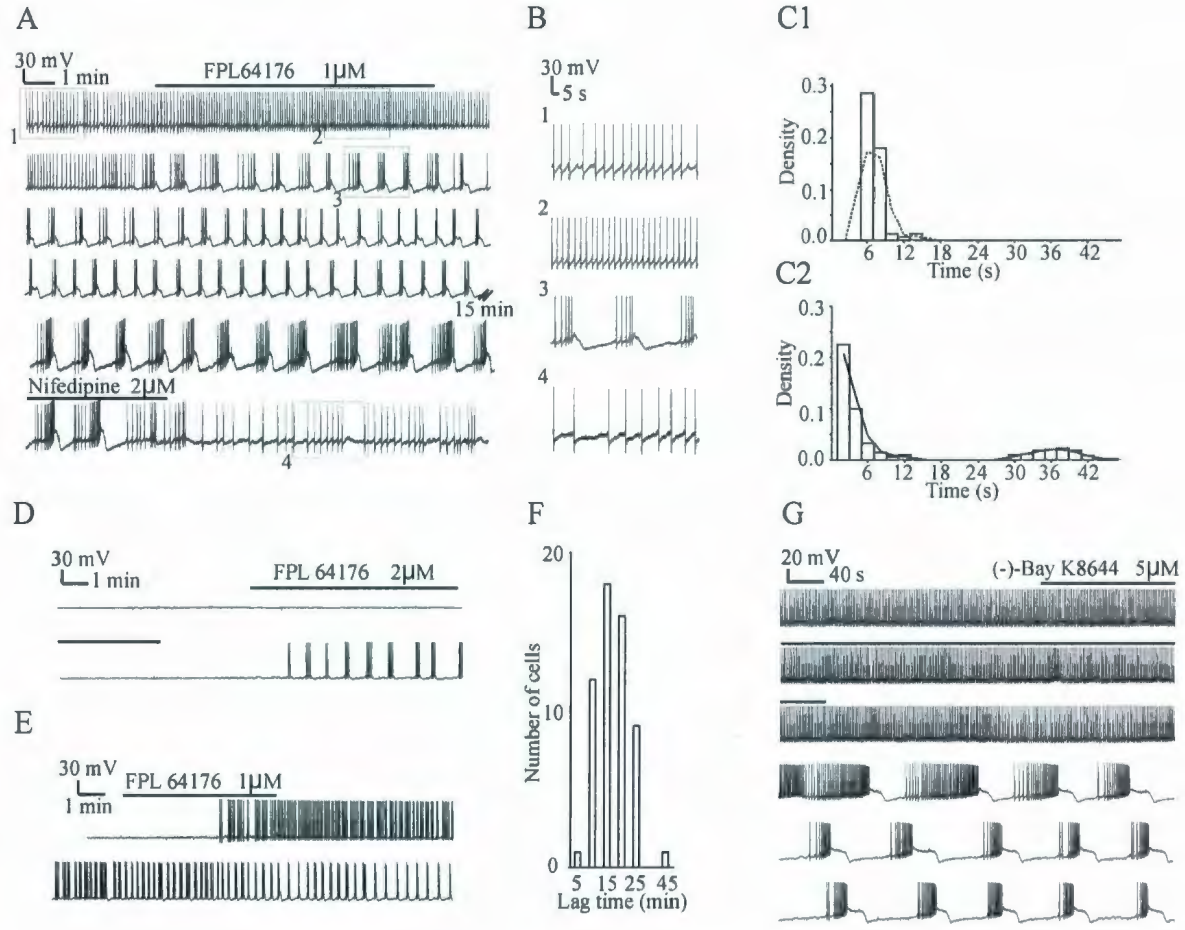

Figure 2.1 FPL 64176 induces burst firing of VTA DA cells. A. Continuous current clamp recording from a representative cell showing that FPL 64176 converted regular firing to burst firing which could be blocked by nifedipine. **B.** Traces on expanded time scales showing regular firing before FPL 64176 application (1), regular firing at higher rates (2) and burst firing after drug application (3) that was brought back to regular firing by nifedipine (4). Note the low frequency of regular firing with a pronounced afterhyperpolarization following each action potential and the clustering of action potentials at increased frequencies followed by a steep post-burst hyperpolarization in burst firing mode. **C.** Density plot of interspike intervals (ISI) in 2-second bins in control conditions (C1, 76 events) and following FPL 64176 application (C2, 246 events). FPL 64176 dramatically shifted the primary peak to the left and gave rise to a secondary peak corresponding to the frequency of burst firing cycles. **D.** Continuous current clamp recording from a representative cell showing that FPL 64176 induced sudden burst firing in a quiescent cell. **E.** Continuous current clamp recording from a representative cell showing that FPL 64176 induced irregular firing that evolved into burst firing in a cell that had no baseline firing. **F.** Histogram of number of cells (n=57) that started burst firing following the onset of FPL 64176 application. **G.** Continuous current clamp recording from a representative cell showing that (S)-(-)-Bay K8644 converted regular firing to burst firing.

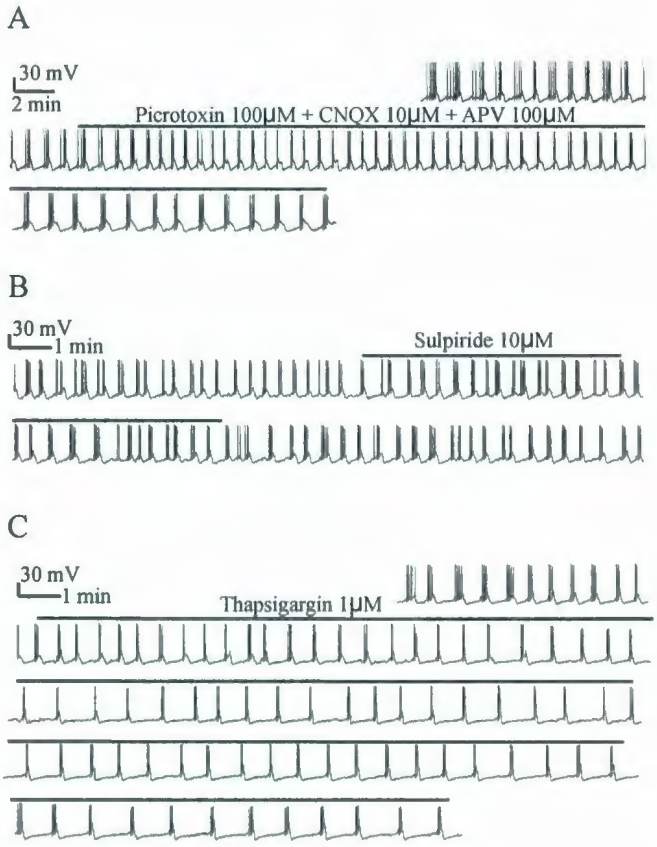

Figure 2.2 FPL 64176 induces burst firing independent of glutamate and GABA ionotropic receptors, D_2 DA receptors and internal Ca^{2+} stores. Continuous current clamp recording from representative cells showing FPL 64176-induced burst firing that persisted in the presence of a cocktail containing blockers at the $GABA_A$, AMPA and NMDA site (**A**), the D_2 antagonist sulpiride (**B**) and the SERCA inhibitor thapsigargin (**C**).

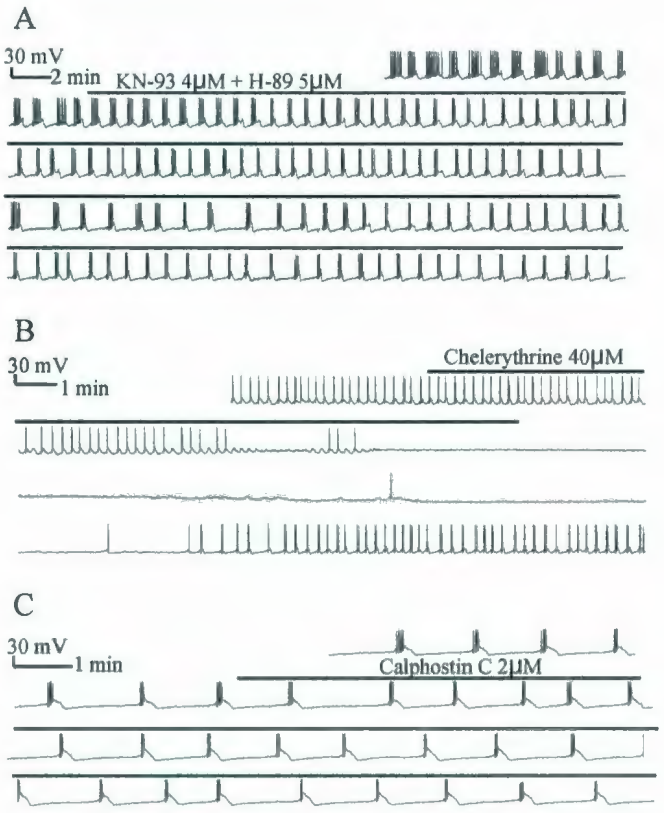

Figure 2.3 PKC mediates FPL 64176-induced burst firing. Continuous current clamp recording from representative cells showing FPL 64176-induced burst firing that persisted in the presence of PKA or CAMKII inhibitors or both (**A**), was reversibly blocked by the substrate site PKC inhibitor chelerythrine (**B**), but not by the PKC blocker that binds to the DAG site in the regulatory subunit (**C**).

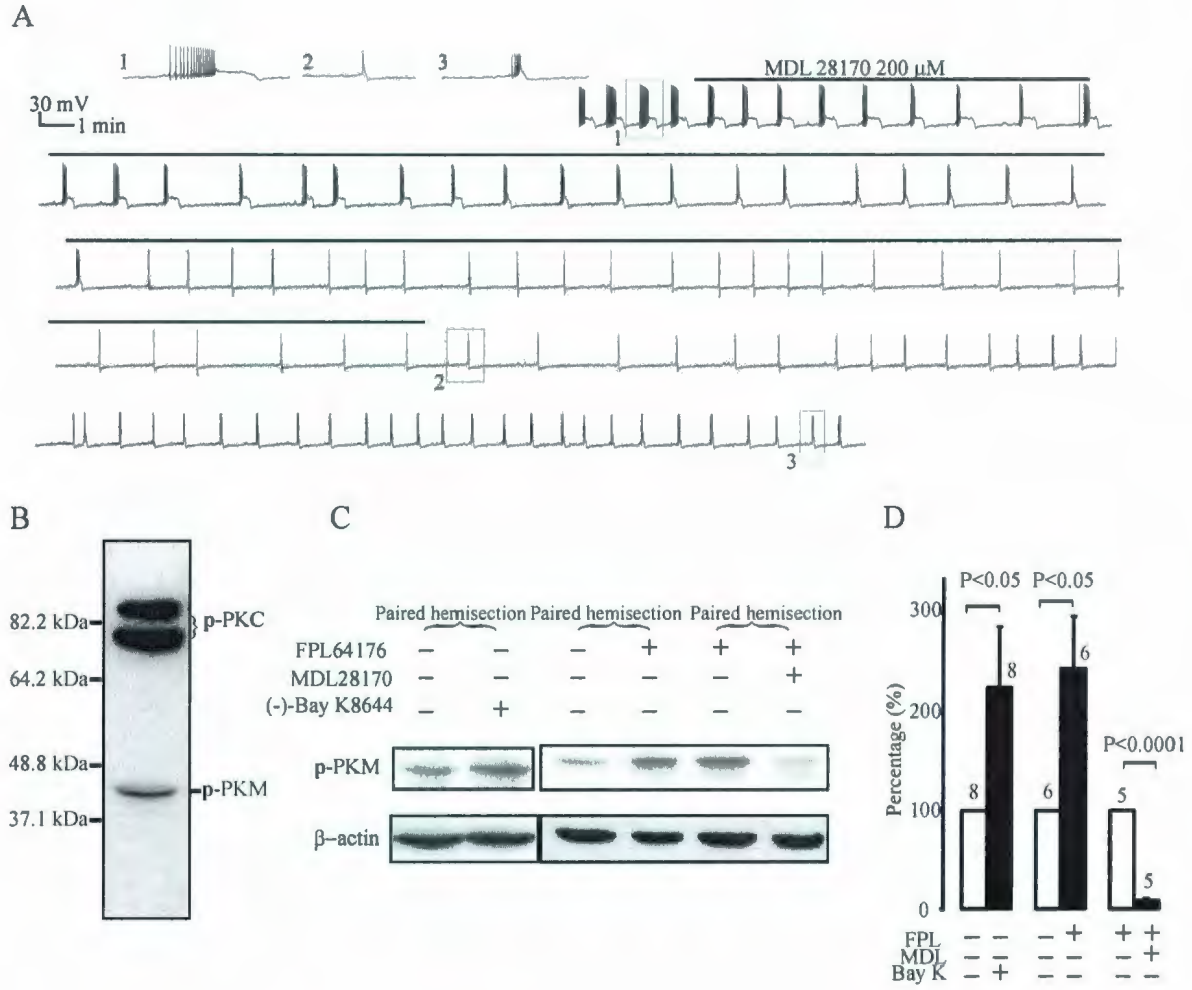

Figure 2.4 Proteolytic cleavage of PKC mediates FPL 64176-induced burst firing. Continuous current clamp recording from a representative cell showing that FPL 64176-induced burst firing was reversibly blocked by the calpain inhibitor MDL 28170 **(A).** Western blots showing phosphorylated PKC isoforms at approximate 82 kDa and a smaller band of PKM at approx 45 kDa **(B).** PKM was increased by FPL 64176 (2 μM, 30 min) or (S)-(-)-Bay K8644 incubation (5 μM, 30 min) and abolished by prior treatment with MDL 28170 (200 μM, 30 min) followed by FPL 64176 (2 μM, 30 min) with MDL 28170 **(C).** Levels of phosphorylated PKM in response to FPL 64176 (n=6,) or (S)-(-)-Bay K8644 (n=8) relative to control slices and PKM levels with prior MDL 28170 treatment relative to FPL 64176 alone (n=5) **(D).** Levels of PKM were normalized against β-actin values obtained from the same blot.

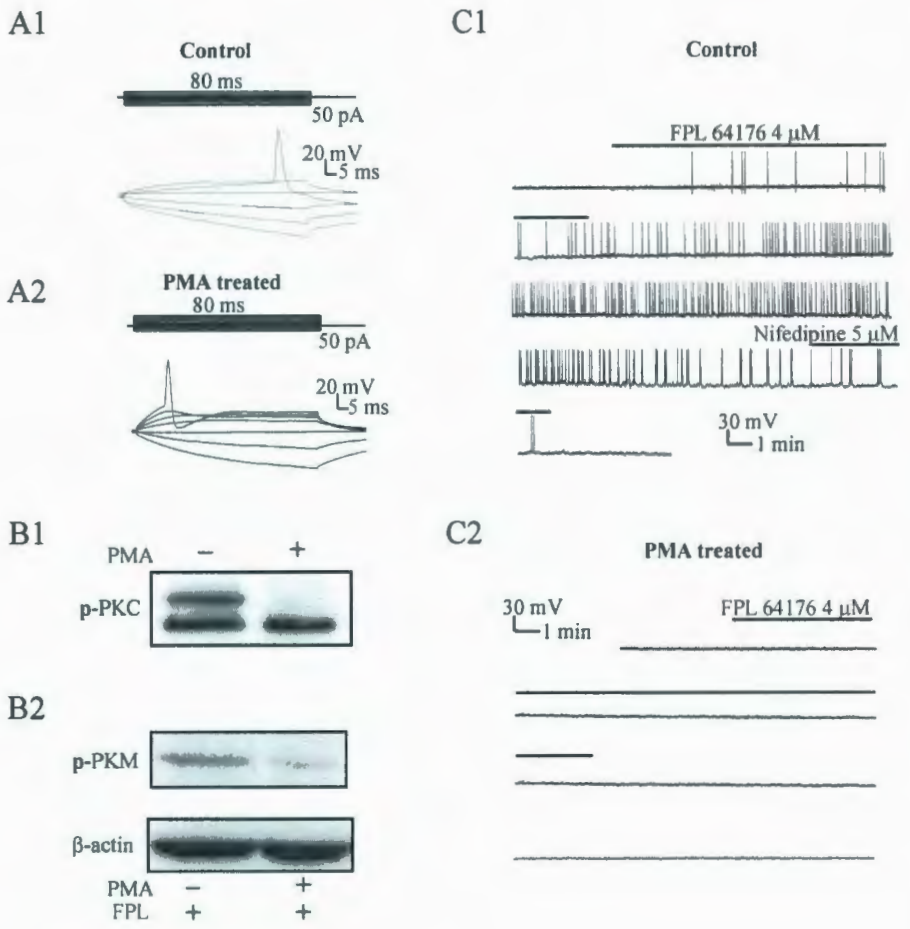

Figure 2.5 FPL 64176 fails to induce burst firing following PKC depletion. **A.** Comparable current-voltage relationships between cells that were incubated in normal ACSF or in PMA 2 μM for 20 h. **B.** Western blots showing a diminished band of phosphorylated PKC isoforms (B1) and a much smaller phosphorylated PKM increase (B2) following FPL 64176 (4 μM, 30 min) in PMA-treated slices (2 μM, 20 h). **C.** Continuous current clamp recording from representative cells showing FPL 64176 induced burst firing that could be blocked by nifedipine in ACSF-treated slices (C1) but not in PMA-treated slices (C2).

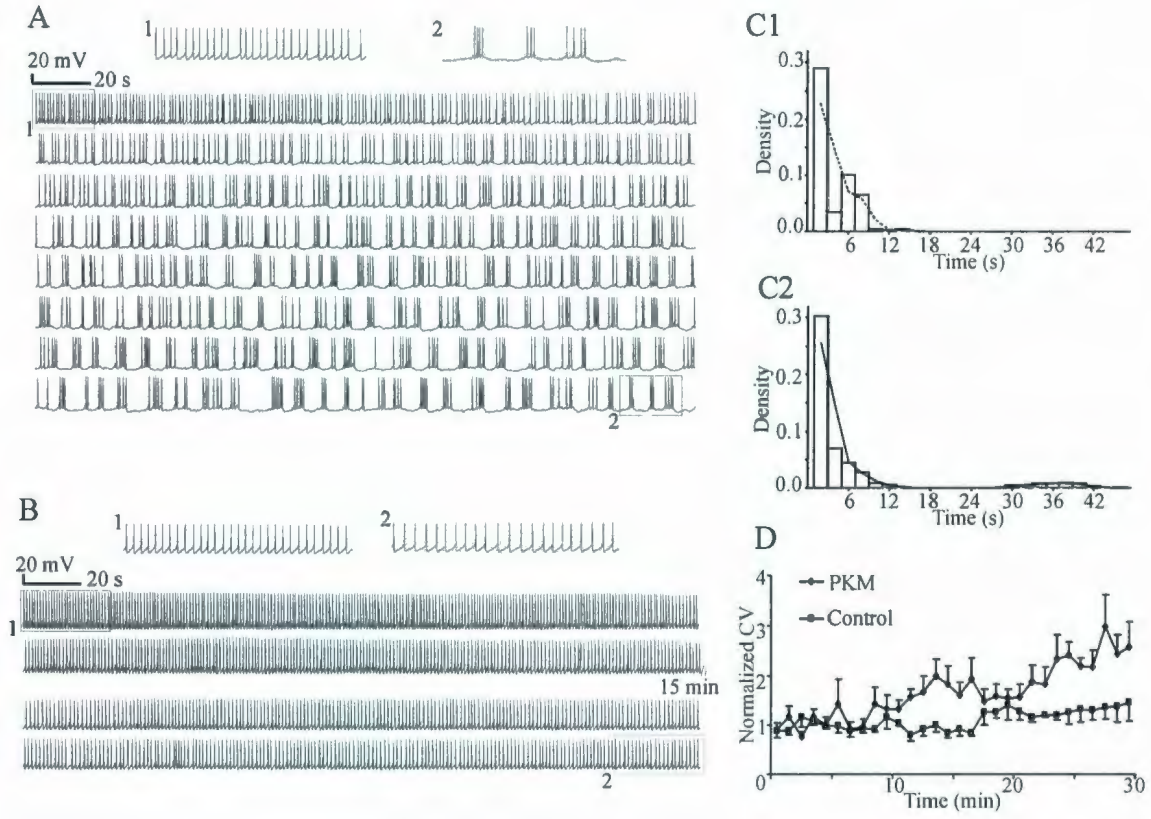

Figure 2.6 Direct loading of purified PKM induces burst firing. A. Continuous current clamp recording from a representative cell showing that PKM loading gradually transformed regular firing into burst firing. Traces on expanded time scales showing regular firing at the beginning (1) and burst firing 30 min after going whole cell (2). **B.** Control whole cell recording showed regular firing throughout the recording period. Traces on expanded time scales showing regular firing at the beginning (1) and 30 min after going whole cell (2). **C.** Density plot of interspike intervals (ISI) in 2-second bins for the first 3 min (C1, 136 events) and 22-35 min (C2, 370 events) following PKM loading. Note the single ISI distribution in the first 3 min (C1) and PKM loading generated a second distribution at lower frequencies (C2). **D.** Normalized coefficient of variance (CV, over 1 min periods) against the average value of the first 5 min in control (n=6) or PKM loading groups (n=7) showing PKM increased CV values.

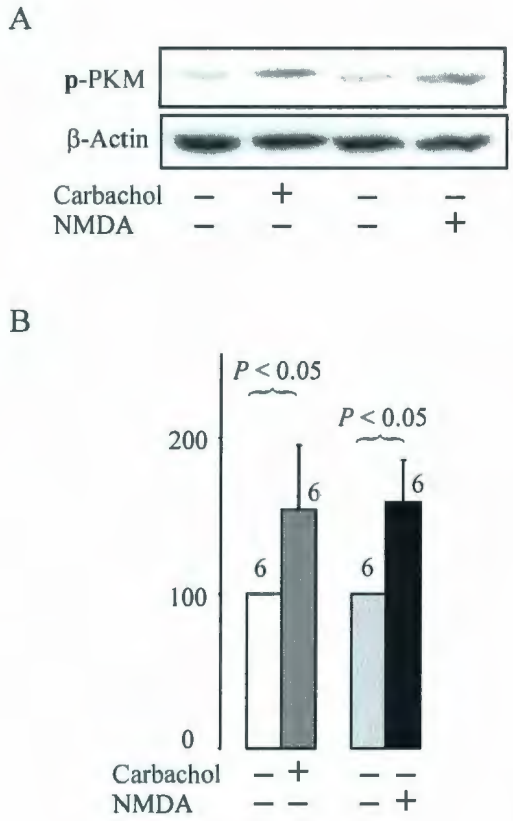

Figure 2.7 NMDA or carbachol induces PKM expression.
A. Western blots showing phosphorylated PKM was increased by NMDA (20 μM, 20 min) or carbachol (20 μM, 20min) incubation.
B. Levels of phosphorylated PKM in response to NMDA (n=6) or carbachol (n=6) relative to control slices.

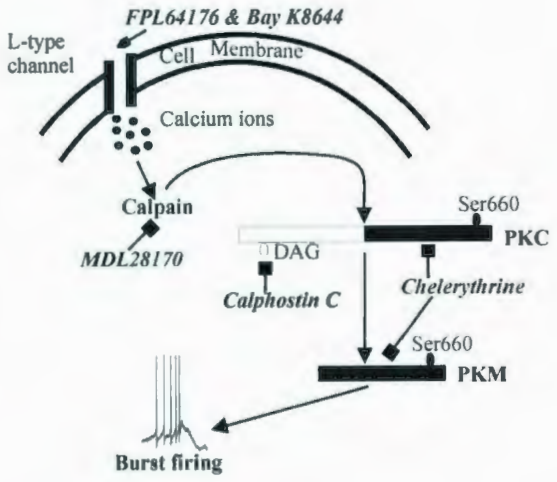

Figure 2.8 Proposed model of PKM-induced bursting.
Schematic representation of the proposed model of PKM-induced bursting, inferred from our results. Calcium entry through L-type channels activates the Ca^{2+}-dependent protease calpain which cleaves PKC into cofactor-independent PKM to induce burst firing in dopamine-responsive neurons in ventral tegmental area.

Chapter 3 Cholinergic Excitation of Dopaminergic Cells Depends on Sequential Activation of Protein Kinase C and the L-type Calcium Channel in Ventral Tegmental Area Slices

(published in Brain Res 1245: 41-51, 2008)

3.1 Introduction

DA cells in the VTA project to the NAc and prefrontal cortex, a circuitry that is commonly known as the reward pathway (Cooper, 2002;Ikemoto, 2007). It has been shown that reward processing is initiated in the VTA which codes for novelty and salient valence of a stimulus in the form of burst firing (Cooper, 2002;Tobler, et al., 2005). The cortical analysis of sensory information is relayed to the VTA through a direct projection from the prefrontal cortex or, indirectly, via its connections to midbrain nuclei, the PPTg and the LDTg. Direct projections from the prefrontal cortex are glutamatergic in nature whereas those via the midbrain nuclei are mixed glutamatergic and cholinergic (Mena-Segovia, et al., 2008;Sesack, et al., 2003). Stimulation of these areas induces large changes in the rate and pattern of firing in the VTA (Cooper, 2002;Grillner and Mercuri, 2002;Kitai, et al., 1999;Lodge and Grace, 2006).

PKC phosphorylates specific serine/threonine sites in a protein to alter its functions. Many proteins are substrates of PKC including glutamatergic and cholinergic receptors as

well as ion channels that are involved in maintaining a cell's excitability (Majewski and Iannazzo, 1998). As a result, PKC in the VTA has been implicated in addiction and motivation. Repeated administration of cocaine increases overall PKC activity in the VTA (Steketee, et al., 1998) whereas injection of PKC inhibitors into the VTA reduces cocaine-induced DA release in the NAc (Steketee, 1993) and delays the onset of behavioral sensitization (Steketee, 1994;1997). Glutamate and acetylcholine are primary excitatory neurotransmitters in the VTA, both of which could activate PKC through their ionotropic (by increasing intracellular Ca^{2+}) and metabotropic receptors (by activating phospholipase C to generate lipid second messengers). It is therefore logical to examine whether PKC is involved in increased VTA firing following activation of these transmitter systems.

L-type calcium channels are responsible for approximately one third of total Ca^{2+} currents (Cardozo and Bean, 1995;Durante, et al., 2004;Takada, et al., 2001) and contribute preferentially to whole-cell Ca^{2+} currents evoked by small depolarization (Durante, et al., 2004;Xu and Lipscombe, 2001) in DA cells. Recently, a dominant role of $Ca_v1.3$, a subtype of the L-type calcium channel that operates at close to resting membrane potentials, was reported in adult mice to sustain pacemaking activities of DA cells (Chan, et al., 2007). Since L-type calcium channels are substrates of PKC and both glutamatergic and cholinergic receptors are coupled to the PKC cascade, it is possible that synaptic modulation of DA firing is due to L-type calcium channel phosphorylation that increases channel opening and conductance (Kamp and Hell, 2000). This is important not

only because of the number of $Ca_v1.3$ channels expressed in DA cells (Chan, et al., 2007;Takada, et al., 2001), but also because of the voltage range at which these channels operate such that their opening, and the modulation of their opening, makes a dominant contribution to the subthreshold depolarization that precede action potential firing.

Previously, we reported that carbachol induces burst firing in a small percentage (20%) of DA cells, longer period of application or higher concentration of carbachol did not increase the percentage of bursting cells, by increasing L-type calcium channel activity (Zhang, et al., 2005). I further reported that Ca^{2+} influx through L-type calcium channel results in PKC cleavage to generate persistently active PKM and induces burst firing (Liu, et al., 2007;Zhang, et al., 2005). However, brief application of cholinergic and glutamatergic agonists only increases the rate of pacemaker firing without the appearance of burst firing in a much bigger portion of DA cells, I therefore examined whether PKC and L-type calcium channels were recruited in excitation induced this way. Here, I present results showing that carbachol activation enhances PKC activity to regulate L-type calcium channels to excite DA cells, while neither NMDA- nor AMPA-induced excitation is dependent on PKC or L-type calcium channels.

3.2 Materials and methods

3.2.1 Slice preparation

The slice preparation was the same as that described in 2.2.1 except that brain was cooled *in situ* with a different ice-cold, carbogenated (95% O_2 and 5% CO_2) cutting solution, in which all NaCl used in ACSF in section 2.2.1 was replaced by equi-osmotic glycerol during slice preparation (Ye, et al., 2006) (composition: 250 mM glycerol, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 26 mM $NaHCO_3$, and 11 mM glucose, pH 7.4 when bubbled with carbogen) after the skull was removed and a block containing the midbrain was cut in this cutting solution, and tissue slices were allowed to recover at 31°C in the same ACSF as that in 2.2.1 for 1 hr instead of recovering at room temperature, and then maintained at room temperature until use. This modified glycerol-based ACSF used only during slice preparation is to prevent the possible acute neurotoxic effects of passive chloride entry, subsequent cell swelling and lysis. Examination of some electrophysiological properties of the neurons in the VTA in these preparations reveals similar properties with the traditional ACSF method (Ye, et al., 2006).

3.2.2 Electrophysiological patch clamp recording

The methods of nystatin-perforated patch clamp recording were the same as that described in 2.2.2.

For recording HVA Ca^{2+} currents, standard whole-cell configuration was used. Patch pipettes were filled with a solution containing 120 mM CsCl, 10mM HEPES, 10 mM EGTA, 4 mM MgATP, 0.3 mM NaGTP. The extracellular solution for HVA Ca^{2+} current recording contained 120 mM TEA-Cl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 18 mM $NaHCO_3$, 11 mM glucose, 3 mM CsCl, 0.3 mM Ni_2Cl, 1 mM 4-AP, 0.3 μM TTX. This solution blocks voltage-gated K^+, Na^+, I_h and the low voltage gated T-type Ca^{2+} currents to isolate HVA currents. The holding potential was -60 mV, and the test pulse for HVA Ca^{2+} current recording was 0 mV for 150 ms. No series resistance compensation or leak subtraction was used in these recordings. Cells whose access resistance and capacitance increased significantly during the course of recording (>20%) were discarded.

3.2.3 Drugs

This part was the same as that described in 2.2.2.

3.2.4 Data analysis

The general electrophysiological data analysis was the same as that described in 2.2.4.

Changes in firing relative to baseline were calculated as instantaneous firing rates divided by the average firing rate of the 5 min stable baseline recording and were plotted as 1-min (for experiments using carbachol) or 30-s (for experiments using NMDA or AMPA) bins. Peak response (the highest bin reading following a treatment), response time (duration of the response) and time to peak were also quantified. HVA Ca^{2+} currents were quantified after subtracting residual currents in the presence of the general Ca^{2+} channel blocker Cd^{2+}. Statistical comparisons of electrophysiological data were performed using paired or unpaired two-tailed Student's t tests, one-way ANOVA or ANOVA for repeated measures (for comparing time-dependent, multiple points between two treatments) as appropriate. To satisfy the normalization test for small sample sizes, all data entries were cubic root transformed before statistical comparisons. Values were considered significant when $P < 0.05$.

3.3 Results

3.3.1 PKC mediates carbachol- but not NMDA- and AMPA-induced excitation

First I examined the role of PKC in carbachol-, NMDA- and AMPA-induced excitation of DA cells. In order to compare the response to carbachol, NMDA and AMPA under different conditions, I initially established that repeated bath application of these ligands induced comparable responses (carbachol at 5 μM for 30 s, n=4, 0.403±0.102 Hz following the first application vs. 0.403±0.148 Hz following a repeated application, P>0.05, paired t test; NMDA at 10 μM for 45 s, n=3, 0.485±0.087 Hz vs. 0.507±0.141 Hz, P>0.05; AMPA at 1 μM for 30 s, n=3, 1.263±0.091 Hz vs. 1.216±0.065 Hz, P>0.05). The response to carbachol, NMDA or AMPA was measured. After full recovery, the PKC blocker chelerythrine was applied (20 μM for at least 20 min) and the response to carbachol, NMDA or AMPA was recorded in the presence of chelerythrine. All three ligands caused a significant and reversible increase in firing rates (0.52±0.65 Hz increased to 1.24±0.65 Hz for carbachol, 0.24±0.15 Hz increased to 1.31±0.42 Hz for NMDA, and 0.5±0.26 Hz increased to 1.38±0.38 Hz for AMPA). Chelerythrine itself caused a significant increased firing (n=16, from 0.27±0.09 Hz to 0.42±0.14 Hz, P<0.05, paired t test) and when this response stabilized, the three ligands were still able to further excite DA cells (0.8±0.27 Hz increased to 1.47±0.76 Hz for carbachol, 0.44±0.24 Hz increased to 1.5±0.46 Hz for NMDA, and 0.19±0.97 Hz increased to 1.46±0.51 Hz for AMPA). However, when relative changes before and after chelerythrine treatments were

compared, only excitation induced by carbachol (Figure 3.1A3) was significantly reduced by the PKC blocker chelerythrine (n=6, $P<0.05$, ANOVA for repeated measures). The effects of NMDA (Figure 3.1B3, n=5, $P>0.05$) and AMPA (Figure 3.1C3, n=5, $P>0.05$) were unaffected by PKC inhibition. Peak response to carbachol (Figure 3.1D) was decreased by 42.8±7.5% in the presence of chelerythrine (n=6, $P<0.05$, paired t test), the decrease was insignificant for NMDA (24.2±9.9%, n=6, $P>0.05$) and there was an increase in the peak response to AMPA which also did not reach significance (42.9±39.3%, n=5, $P>0.05$). Chelerythrine did not change the response time (Figure 3.1E) or time to peak for any of the three ligands.

To verify that chelerythrine decreased carbachol's efficacy by inhibiting PKC, another PKC inhibitor that binds to a different site was used. Similar to chelerythrine, GF 109203X itself caused a significant increase in firing (n=13, from 0.52±0.18 Hz to 0.94±0.36 Hz, $P<0.05$, paired t test). GF 109203X applied at 2 μM for at least 20 min also reduced carbachol-induced excitation (0.65±0.29 increased to 1.16±0.4 Hz for carbachol alone, and 1.17±0.4 increased to 1.61±0.68 Hz for carbachol in the presence of GF 109203X). GF 109203X reduced both the overall response (n=7, $P<0.05$, ANOVA for repeated measures) and the peak response to carbachol (decreased by 33.7±0.10% in the presence of GF 109203X, n=7, $P<0.05$, paired t test).

3.3.2 L-type calcium channels are involved in carbachol- but not NMDA- or AMPA-induced excitation.

The L-type calcium channel is abundantly expressed in DA cells (Takada, et al., 2001) which can be recruited by cholinergic and glutamatergic agonists to increase excitability either by direct depolarization or by PKC-mediated phosphorylation. Similarly to the initial experiments, carbachol (5 μM for 30s), NMDA (10 μM for 45s) or AMPA (1 μM for 30s) was first applied and the response was allowed to recover; then the specific L-type calcium channel blocker nifedipine (5 μM) was applied for 8 min and the response to carbachol, NMDA or AMPA was recorded again in the presence of nifedipine. Nifedipine itself did not change firing rates significantly in the cells tested (n=20, peak response before and after application of nifedipine: 0.37±0.09 Hz vs. 0.44±0.11 Hz, $P>0.05$, paired t test). Figure 3.2 shows that the carbachol-induced increase (Figure 3.2A3) in firing rates was also significantly reduced by nifedipine (n=5, 0.5±0.23 increased to 1.0±0.35 Hz for carbachol alone vs. 0.52±0.23 increased to 0.76±0.34 Hz for carbachol in the presence of nifedipine, $P<0.05$, ANOVA for repeated measures), however, increased firing following NMDA (Figure 3.2B3, n=7, $P>0.05$) or AMPA (Figure 3.2C3, n=7, $P>0.05$) was unaffected by nifedipine (0.3±0.12 increased to 1.52±0.33 Hz for NMDA alone vs. 0.39±0.19 increased to 1.68±0.49 Hz for NMDA in the presence of nifedipine; 0.34±0.15 increased to 1.61±0.4 Hz for AMPA alone vs. 0.43±0.21 increased to 1.67±0.58 Hz for AMPA in the presence of nifedipine). Peak response (Figure 3.2D) to carbachol was significantly decreased by 34.7±9.4% in the

presence of nifedipine (n=5, P<0.05, paired t test), but peak responses to NMDA (decrease by 1.4±11.0%, n=8, P>0.05) or AMPA (decreased by 19.6±14.3%, n=7, P>0.05) were unchanged. Nifedipine did not change the response time (Figure 3.2E) or time to peak for any of the three ligands.

3.3.3 PKC and L-type calcium channel blockade are not additional in suppressing carbachol-induced excitation

Since both PKC and L-type calcium channels were involved in carbachol-induced excitation, I asked whether the two were related events of the same cascade. If PKC and L-type calcium channels were sequentially activated by carbachol, it would be expected that the combined application of both a PKC inhibitor and L-type calcium channel blocker would produce no more suppression than the same PKC inhibitor or L-type calcium channel blocker alone. Carbachol (5 μM for 30s) was first applied and the response was allowed to recover; then a cocktail containing 5 μM nifedipine and 20 μM chelerythrine was applied for 15 min and the response to carbachol was recorded again in the presence of the cocktail. The cocktail itself did not change the firing rates in the cells (n=6, 0.38±0.08 Hz vs. 0.20±0.08 Hz, P>0.05, paired t test). Carbachol increased firing from 0.38±0.08 Hz to 0.84±0.11 Hz and this increase was smaller in the presence of the cocktail (from 0.2±0.08 Hz to 0.34±0.1 Hz). Figure 3.3 shows that the carbachol-induced fold increase in firing was significantly reduced in the presence of the cocktail (Figure 3.3C, n=6, P<0.05, ANOVA for repeated measures). The peak response (Figure 3.3D)

was also significantly decreased by 41.0±13.2% in the presence of the cocktail (n=6, $P<0.05$, paired t test). The response time (Figure 3.3E) and time to peak were not significantly changed by combined application of the PKC inhibitor and L-type calcium channel blocker.

Relative increases in firing rates following carbachol combined with chelerythrine or nifedipine, or both as a percentage of increased rates induced by carbachol alone obtained in their respective control groups were plotted to assess whether chelerythrine, nifedipine or their combined application altered carbachol's actions to the same extent. There were no significant differences among the three treatment groups: carbachol with chelerythrine (n=6), nifedipine (n=5) or both (n=6) (Figure 3.3F, $P>0.05$, ANOVA for repeated measures). Peak responses to carbachol in these three groups (Figure 3.3G) were not significantly different ($P>0.05$, one-way ANOVA).

3.3.4 Carbachol increases nifedipine-sensitive L-type calcium currents by activating PKC

Inhibiting PKC and blocking L-type calcium channels reduced carbachol-induced excitation to the same extent and combined application of both did not produce additive effects. This suggests that PKC activation and L-type calcium channel opening are sequential steps in the same cascade. To examine how L-type calcium currents are altered by carbachol and PKC inhibition, I isolated HVA Ca^{2+} currents which were blockable by

Cd^{2+}. Three parallel groups were set up to record HVA Ca^{2+} currents continuously at 30 s intervals. The control group consisted of a 5 min baseline recording and 7 min in 10 μM nifedipine followed by 5 min in 100 μM Cd^{2+}. The carbachol group consisted of 5 min in 20 μM carbachol, 7 min in 10 μM nifedipine and 5 min in 100 μM Cd^{2+}. For PKC inhibition, slices were first incubated in 1 μM GF 109203X for at least 40 min, and were continuously perfused with 1 μM GF 109203X during all recordings which were the same as those of the carbachol group. All currents were normalized by subtracting residual currents after a 5-min Cd^{2+} application. In 7 control cells, total HVA Ca^{2+} currents were decreased by 5.3±2.0% in the first 5 min due to current run-down and nifedipine blocked 13.7±3.1% at the 12^{th} minute. In eight carbachol-treated cells, total currents increased by 0.6±1.9% in the first 5 min and nifedipine blocked 25.3±4.3% of the currents. In the 9 cells with PKC inhibition, carbachol decreased the total currents by 3.5±4.8% in the first 5 min, and nifedipine blocked 9.5±2.3% of the currents. Carbachol induced a minimal increase in the currents just enough to overcome the run-down effects (Figure 3.4B, $P>0.05$, one-way ANOVA). However, carbachol significantly changed the nifedipine-sensitive portion of the currents (Figure 3.4C, $P<0.05$, one-way ANOVA). Post hoc tests (least significant difference method, LSD) showed that carbachol significantly increased the percentage of nifedipine-sensitive Ca^{2+} currents ($P<0.05$) and PKC inhibition significantly blunted the carbachol-induced increase in nifedipine-sensitive Ca^{2+} currents ($P<0.05$). There was no significant difference between the control and PKC inhibition groups ($P>0.05$).

3.4 Discussion

Firing behavior of DA cells in the VTA is related to DA transmission associated with normal or abnormal expression of motivation and reward processing. VTA-DA cell firing is modulated by synaptic activity (Kitai, et al., 1999). In this study, I found that carbachol, but not NMDA or AMPA, induced increased firing by activating PKC and L-type calcium channels. I further found that carbachol increased the nifedipine-sensitive portion of the HVA Ca^{2+} currents, an effect that was also blocked by PKC inhibition. My results indicate that cholinergic, but not glutamatergic, activation leads to a positive modulation of L-type calcium channels by PKC to mediate increased firing of DA cells in the VTA.

3.4.1 Carbachol activates PKC to up-regulate L-type calcium channels to induce excitation

My results demonstrate that carbachol-induced excitation of DA cells was dependent on PKC activation and L-type calcium channel opening. These two events affected carbachol's effects to the same extent and they were not additive suggesting that they are sequential to one another. Carbachol has the capability of activating PKC and the L-type calcium channels through multiple pathways. It can activate PKC by an increase in intracellular Ca^{2+} by way of nAChR-mediated Ca^{2+} influx or mAChR-mediated Ca^{2+} release from internal Ca^{2+} stores. Additionally, mAChRs are coupled to phospholipase C

to generate lipid signals such as inositol (1, 4, 5) trisphosphate (IP_3) and DAG which activate PKC. At the same time, carbachol depolarizes the membrane, which can activate L-type calcium channels especially the $Ca_v1.3$ subtype that operates near resting membrane potentials. Increased spiking, as the result of this depolarization, will bring the majority of voltage-gated channels including all types of Ca^{2+} channels into play. The question is how carbachol activates the two events sequentially.

Depolarization and increased firing are unlikely the initial steps of this sequence of events because in my series NMDA and AMPA caused similar depolarization and increased spiking which could not be significantly reduced by PKC inhibition and L-type calcium channel blockade. This strengthens the other possibility that carbachol activates PKC which in turn upregulates the L-type calcium channel to increase spiking activity of the DA cells. PKC does phosphorylate both subtypes of the L-type calcium channel at multiple serine/threonine sites. It has been shown that phosphorylation of the $Ca_v1.2$ by PKC leads to positive modulation of the channel (Yang, et al., 2005) so that it becomes easier to open and conducts more Ca^{2+} ions. PKC phosphorylation of the $Ca_v1.3$ subtype has been less intensely studied. Some report that PKC phosphorylation of the $Ca_v1.3$ subtype results in negative modulation of the channel (Chahine, et al., 2008), while others contend that only phosphorylation of the N-terminal produces this effect (Baroudi, et al., 2006). Positive modulation of L-type calcium channels by PKC has been reported in native tissues. For example, M_2 mAChR stimulation requires PKC to increase $Ca_v1.2$ currents in rabbit portal vein myocytes (Callaghan, et al., 2004) and M_1 receptor

activation involves PKC to mediate facilitation of L-type calcium channels in dissociated autonomic neurons from the major pelvic ganglion (Sculptoreanu, et al., 2001). PKC-mediated facilitation of L-type calcium channels has not been previously demonstrated in midbrain DA cells, so my results that PKC inhibitors reduced carbachol-induced increases in nifedipine-sensitive Ca^{2+} currents clearly show that such a modulation exists in DA cells.

3.4.2 NMDA- or AMPA-induced excitation does not activate PKC to excite DA cells.

NMDA receptor and AMPA receptors with GluR2 subunit composition are permeable to Ca^{2+} and they undoubtedly depolarize the membrane and increase the frequency of spiking which promotes Ca^{2+} influx through voltage-gated Ca^{2+} channels. However, our results clearly show that both glutamatergic agonists did not cause a PKC-mediated facilitation of L-type calcium channels to bring about their excitatory actions. My previous study shows that NMDA increases the production of PKM (the persistently active catalytic subunit of PKC) in VTA slices (Liu, et al., 2007), a cascade that is required for converting pacemaking firing to bursting in DA cells. Why did PKC inhibition fail to alter NMDA-mediated excitation? The crucial difference between this paper and our previous results is that in this series I applied NMDA at a lower concentration (10 μM) for only a brief period (45 s) so that the response could be washed out completely within a relatively short time to retest the response in the presence of PKC inhibition. In our previous work, however, I used a much higher dose (20 μM) for a more

prolonged period of time (20 min) to maximize PKM expression. This argues against a role of PKM generation to mediate NMDA-induced firing at this particular concentration, however, it does not explain the lack of PKC participation in this process. I think that the discrepancy in PKC reliance following cholinergic and glutamatergic agonists might arise from the following possibilities.

Firstly, carbachol activates nAChRs and mAChRs with a highly probable signaling convergence on PKC as both receptors are known to increase Ca^{2+} and lipid signals that are activators of this kinase. While NMDA and to a lesser extent AMPA do increase intracellular Ca^{2+} concentrations, this might not be enough for maximal PKC activation because of the fact that Ca^{2+} and lipid second messengers activate PKC synergistically (Hug and Sarre, 1993). Secondly, glutamatergic transmission is mostly synaptic while cholinergic terminals utilize volume transmission (Pickel, et al., 2002). As a result, NMDA and AMPA receptors are clustered at synaptic sites in distal dendrites while cholinoceptors are more widespread throughout the geometry of the cell mostly in the soma and dendrites (Gahring, et al., 2004;Klein and Yakel, 2006;Lena, et al., 1999;Severance and Cuevas, 2004). Since L-type calcium channels are more heavily expressed in the cell body and proximal dendrites (Hell, et al., 1993;Takada, et al., 2001), cholinoceptors are physically closer to these channels to allow a more ready interaction. Thirdly, it has been shown that there exists a signaling scaffold complex of NMDA or AMPA receptors, PKC and calcineurin (Gomez, et al., 2002). Calcineurin dephophorylates serine/threonine and is an important regulator of protein phosphorylation

in DA cells. It is also Ca^{2+}-dependent, requiring a lower Ca^{2+} concentration to activate than that required by PKC (Dash, et al., 2007). The net result following NMDA/AMPA receptor activation might be in favor of calcineurin. However, our results do not exclude the possibility of recruiting PKC by metabotropic glutamate receptors in the real glutamatergic transmission that can activate both ionotropic and metabotropic glutamate receptors. Furthermore, it might be argued that bath application of NMDA/AMPA activates all receptors on a cell whereas in real life, glutamatergic transmission is mostly carried by synaptic NMDA/AMPA receptors. Since PKC phosphorylation of NMDA/AMPA receptors affects their membrane trafficking dynamics by inserting more receptors at the synaptic site (Grosshans, et al., 2002), PKC might affect synaptic transmission more in behaving animals than in slices where drugs in the bath are allowed to diffuse to all receptors.

3.4.3 The significance of cholinoceptor-mediated PKC facilitation of L-type calcium channels

The VTA and its projections to the NAc and the prefrontal cortex are viewed as the final common pathway to all forms of addiction. Nicotine is arguably the most addictive substance abused on a large scale. There is solid evidence that nAChRs on synaptic terminals increase glutamate release, promote the formation of long-term potentiation at glutamatergic synapses and long-term depression at GABAergic synapses in the VTA (Kauer and Malenka, 2007), all of these leading to increased activity of DA cells.

However, it has also been known that the $\alpha_4\beta_2$ nAChRs expressed in postsynaptic DA cells are implicated in regulating the firing activities of DA cells (Sorenson, et al., 1998) and behavioral sensitization (Tapper, et al., 2004). This paper shows that, besides synaptic modulation by nAChRs, nicotine can activate PKC in the postsynaptic cell that facilitates L-type calcium channel opening to underlie addictive behavior. Both PKC and L-type calcium channels have been shown to be implicated in addiction and motivation. For example, repeated administration of cocaine increases overall PKC activity in the VTA (Steketee, et al., 1998), while injection of PKC inhibitors into the VTA reduces cocaine-induced DA release in the NAc (Steketee, 1993) and delays the onset of behavioral sensitization (Steketee, 1994;1997). Similarly, repeated amphetamine injections increase the expression of α1C L-type calcium channel subunits in the VTA (Rajadhyaksha, et al., 2004) and repeated stimulation of L-type calcium channels in the VTA mimics the initiation of behavioral sensitization to cocaine (Licata, et al., 2000), while direct injection of L-type calcium channel antagonists into the VTA attenuates the development of psychostimulant-induced behavioral sensitization (Licata, et al., 2004). These behavioral observations could possibly be explained by our findings in this paper that cholinergic activation leads to PKC activation and L-type calcium channel facilitation, which could drive up the DA firing that is required for behavioral sensitization.

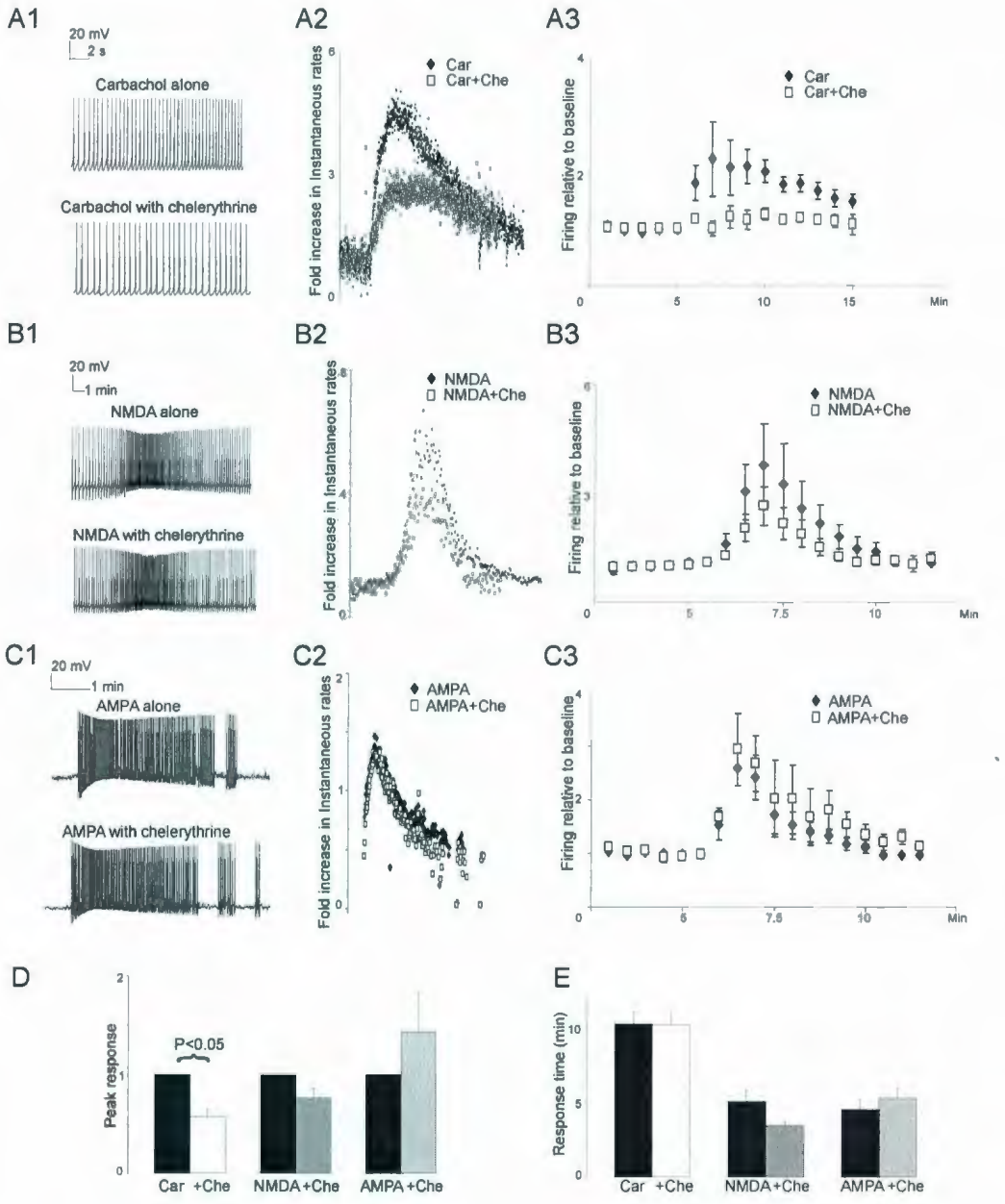

Figure 3.1 PKC mediates carbachol- but not NMDA- and AMPA-induced excitation. **A1-C1.** Current clamp recording from representative cells showing carbachol- (A1, 5 μM), NMDA- (B1, 10 μM) or AMPA-induced (C1, 1 μM) increases firing rates in control conditions and in the presence of the PKC blocker chelethrine (20 μM). **A2-C2.** Relative changes in instantaneous firing frequencies following carbachol (A2), NMDA (B2) or AMPA (C2) from the same cells as in A1-C1 with (open) or without (filled) chelerythrine. **A3-C3.** Percentage changes in firing rates by carbachol (A3, n=6), NMDA (B3, n=5) or AMPA (C3, n=5) with (open) or without (filled) chelerythrine. **D.** Peak responses to carbachol (Car), NMDA or AMPA with chelerythrine (Che) relative to carbachol, NMDA or AMPA alone. **E.** Response time to carbachol (Car), NMDA or AMPA with chelerythrine (Che) relative to carbachol, NMDA or AMPA alone.

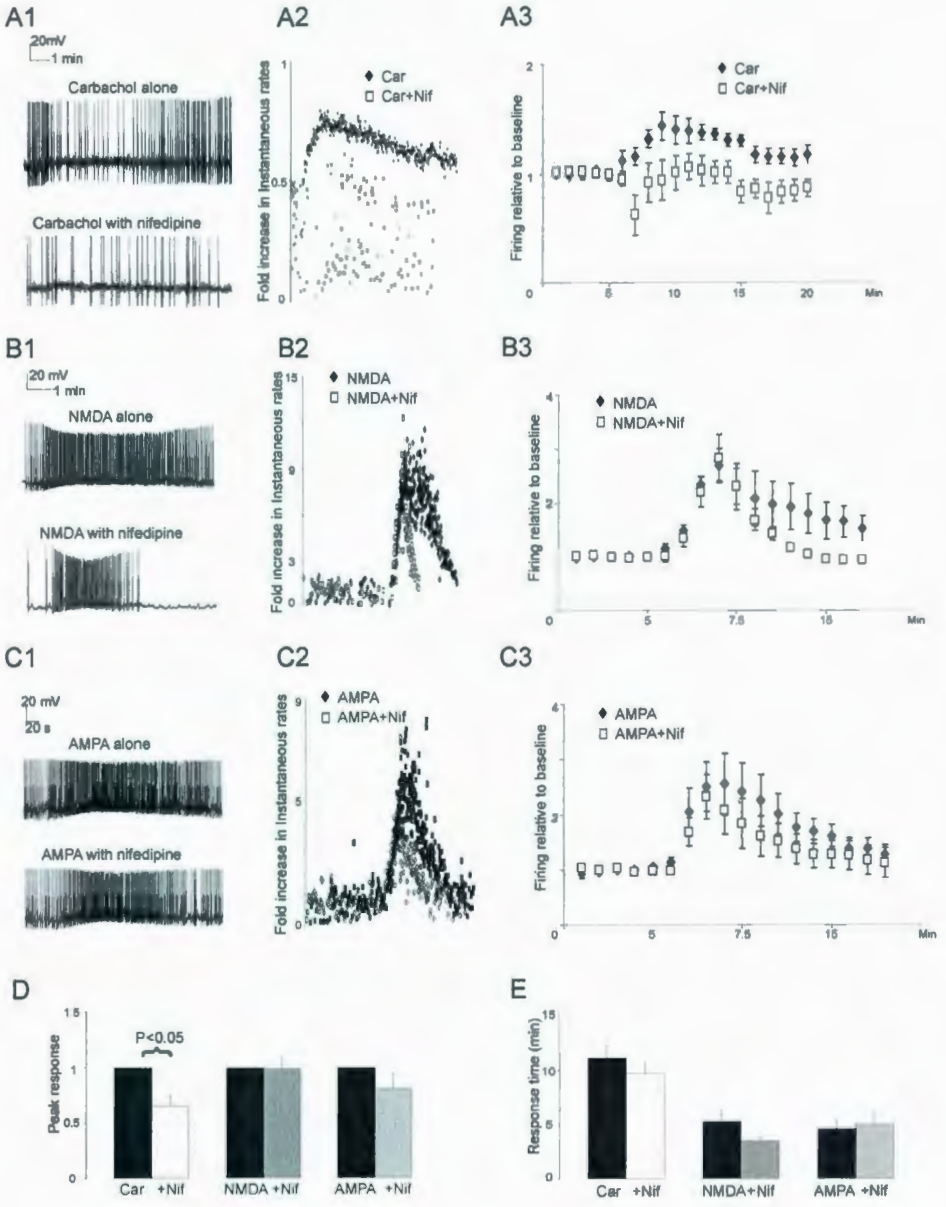

Figure 3.2 L-type Ca^{2+} channels are involved in carbachol- but not NMDA- or AMPA-induced excitation. A1-C1. Current clamp recording from representative cells showing carbachol- (A1, 5 μM), NMDA- (B1, 10 μM) or AMPA-induced (C1, 1 μM) increases in firing rates in control conditions and in the presence of the L-type Ca^{2+} channel blocker nifedipine (5 μM). **A2-C2.** Relative changes in instantaneous firing frequencies by carbachol (A2), NMDA (B2) or AMPA (C2) from the same cells as in A1-C1 with (open) or without (filled) nifedipine. **A3-C3.** Percentage changes in firing rates by carbachol (A3, n=5), NMDA (B3, n=7) or AMPA (C3, n=7) with (open) or without (filled) nifedipine. **D.** Peak responses to carbachol (Car), NMDA or AMPA with nifedipine (Nif) relative to carbachol, NMDA or AMPA alone. **E.** Response time to carbachol (Car), NMDA or AMPA with nifedipine (Nif) relative to carbachol, NMDA or AMPA alone.

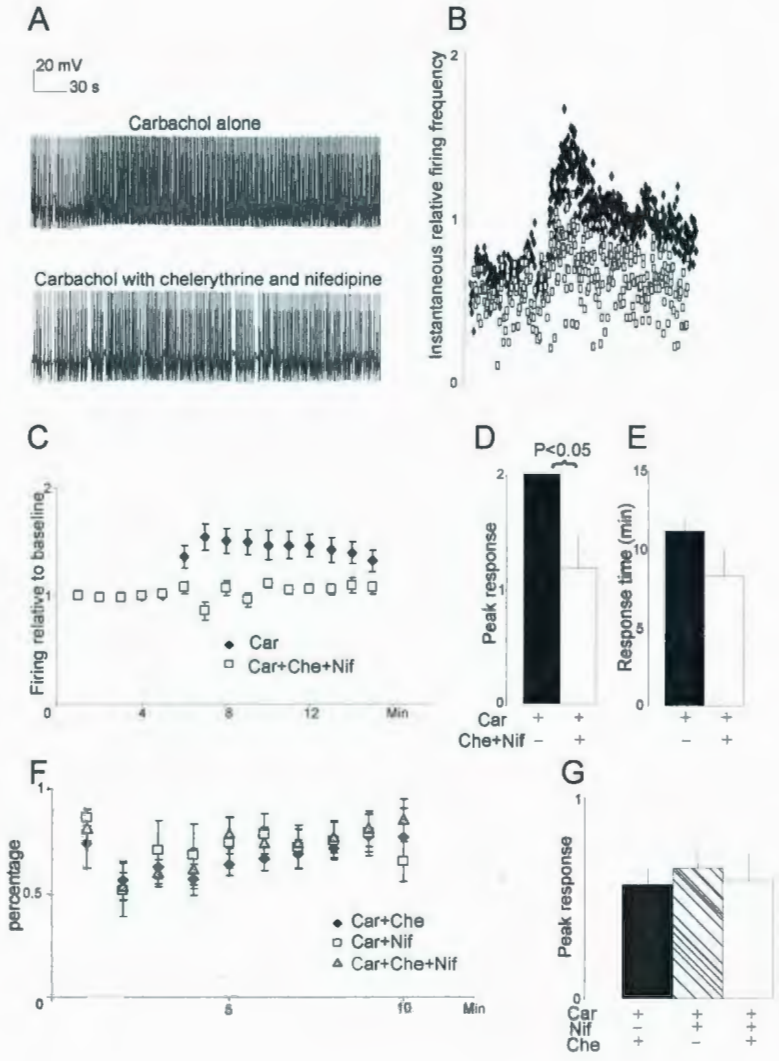

Figure 3.3 PKC and L-type channel blockade are not additional in suppressing carbachol-induced excitation. A. Current clamp recording from a representative cell showing a carbachol-induced (5 μM) increase in firing rates in control conditions and in the presence of a cocktail containing both a PKC inhibitor (chelerythrine, Che, 20 μM) and an L-type Ca^{2+} channel blocker (nifedipine, Nif, 5 μM). **B.** Relative changes in instantaneous firing frequencies by carbachol from the same cell as in A with (open) or without (filled) the cocktail. **C.** Percentage changes in firing rates by carbachol (n=6) with (open) or without (filled) the cocktail. **D.** Peak response to carbachol (Car) with the cocktail relative to carbachol alone. **E.** Response time to carbachol with the cocktail relative to carbachol alone. **F.** Relative increases in firing rates following carbachol (Car) combined with chelerythrine (Che, n=6), nifedipine (Nif, n=5) or both (n=6) as a percentage of increased rates induced by carbachol alone obtained in their respective control groups were plotted to compare whether chelerythrine, nifedipine or their combined application altered carbachol's actions to the same extent. **G.** Peak responses of the three groups in F.

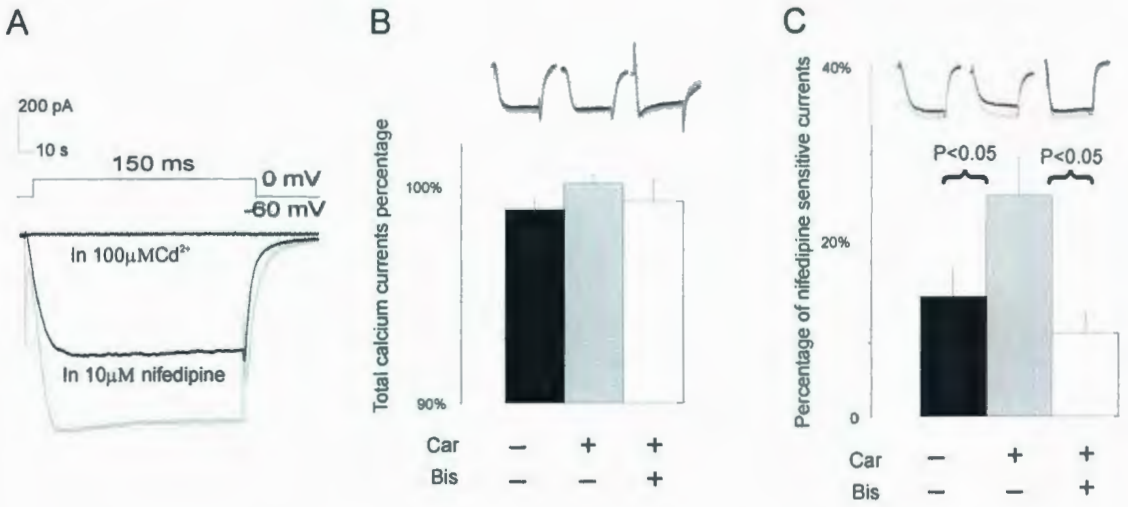

Figure 3.4 Carbachol increases nifedipine-sensitive L-type Ca^{2+} currents by activating PKC. A. Currents evoked in a representative neuron by stepping the membrane potential from -60 mV to 0 mV for 150 ms under voltage-clamp conditions. **B.** Changes in total HVA currents as a percentage of the initial current magnitude in control (n=7), carbachol (Car, 20 μM) alone (n=8) or carbachol in the presence of the PKC inhibitor GF 109203X (Bis, 1 μM, n=9). Insets are HVA current traces at the beginning of recording (grey line) and 5 min after (black line) treatment. **C.** Percentage of nifedipine-sensitive currents for these three groups. Insets are HVA current traces before (grey line) and after nifedipine (black line, 10 μM) application in treated groups shown at the bottom.

Chapter 4 $Ca_v1.3$ L-type calcium Channels Mediate Single Spike and Burst Firing of VTA DA Neurons in Mice

4.1 Introduction

As detailed in Chapter 1, DA neurons in the VTA play an important role in cognition and reward processing (Cooper, 2002;Ikemoto, 2007), and abnormalities in these processes are implicated in such disease states as schizophrenia and drug addiction (Kelley, 2004;Koob, 2000;Seamans and Yang, 2004). DA neurons display two major firing patterns: single spike and burst firing (Grace and Bunney, 1984a;b;Zhang, et al., 2005). The two firing modes have been shown to be involved in different functions: pace-making spiking encodes expected stimulations while burst firing signals unexpected environmental stimuli with reward value related to reward-based learning (Schultz, 1997;1998;Tobler, et al., 2005). Therefore, it is crucial to better understand the transition and regulation of the two firing modes in DA neurons.

Ca^{2+} influx through L-type calcium channels is an important modulator of neuronal firing activities including DA neurons in the VTA. L-type calcium channels are responsible for approximately one third of total Ca^{2+} currents (Cardozo and Bean, 1995;Durante, et al., 2004) and contribute preferentially to whole-cell Ca^{2+} currents evoked by small depolarizations (Durante, et al., 2004;Xu and Lipscombe, 2001) in DA cells. They have been shown to be involved in spontaneous firing, spontaneous

oscillatory potentials and cholinergic-driven firing increases that are abolished by DHP antagonists (Mercuri, et al., 1994;Nedergaard, et al., 1993;Zhang, et al., 2005). Also, they are involved in the regulation of burst firing. For example, the cholinergic-driven bursting and associated membrane potential oscillations are blocked by the L-type calcium channel blocker nifedipine (Zhang, et al., 2005). Furthermore, direct activation of L-type calcium channels with two different activators, (S)-(-)-Bay K8644 and FPL 64176, induces burst firing in DA neurons (Liu, et al., 2007;Zhang, et al., 2005).

Four subtypes of L-type calcium channels haven been identified: $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$ and $Ca_v1.4$; only $Ca_v1.2$ and $Ca_v1.3$ subtypes are expressed in the midbrain (Takada, et al., 2001). It has been shown from immunohistochemical experiments that $Ca_v1.2$ and $Ca_v1.3$ L-type calcium channels are distributed throughout the entire midbrain, including DA neurons (Takada, et al., 2001). Takada et al (Takada, et al., 2001) indicates that DA neurons display more intense immunoreactivity against $Ca_v1.3$ than $Ca_v1.2$ L-channels, but data from our lab (unpublished by Tara Deemayd), using the same method as what Takada used, did not show a dominant expression of $Ca_v1.3$ L-type calcium channels in TH-positive midbrain neurons. As detailed in Chapter 1, $Ca_v1.3$ L-type calcium channels display atypical properties of L-type calcium channels compared to the $Ca_v1.2$ subtype: They have a low activation threshold and a reduced sensitivity to DHP agonists and antagonists (Durante, et al., 2004;Xu and Lipscombe, 2001). The role of L-type calcium channels has been clearly explored but because current pharmacological agents can not isolate $Ca_v1.2$ and $Ca_v1.3$ subtypes, their specific

contribution to functions mediated by L-type calcium channels is still unknown. This chapter reports work aimed to determine the contribution of the two L-type calcium channel subtypes to the firing activities of DA neurons in the VTA by using a knock-in mouse strain carrying a mutant DHP site in the $Ca_v1.2$ subtype.

4.2 Methods

4.2.1 Animals

Wild-type (WT) mice (C57BL/6) (2-9 months old) and transgenic mice carrying a mutant DHP site in α1-subunit of $Ca_v1.2$ L-type calcium channels ($Ca_v1.2DHP^{(-/-)}$, with C57BL/6 genetic background) were used for this study. Point mutation of the DHP site dramatically reduces the sensitivity of $Ca_v1.2$ L-type calcium channels to DHP modulators (Striessnig, et al., 2006;Zhang, et al., 2007). Animal housing was the same as that described in Chapter two. Congenic knock-in mice were first bred from $Ca_v1.2DHP^{(+/-)}$-$Ca_v1.2DHP^{(+/-)}$ pairs and maintained with $Ca_v1.2DHP^{(-/-)}$-$Ca_v1.2DHP^{(-/-)}$ breeding pairs. Mice were ear-tagged and weaned when they were 21 days old and a segment of tail (about 1 cm) was cut for genotyping. Bleeding was generally minor and could be readily stopped by silver nitrate.

4.2.2 Genotyping of the offspring

Mouse tail tips were placed in 1.5 mL microfuge tubes which were filled with 600 µL Lysis buffer/ Proteinase K solution (composition: 99 mM Tris-HCl pH 8.0, 4.95 mM EDTA pH 8.0, 0.198% SDS, 0.198 M NaCl, 0.02% proteinase K). They were left on a rotator at 50 °C overnight and then centrifuged at 15,000 g for 30 min. The aqueous phase was decanted into a new tube, followed by adding 1 mL 100% ethanol and shaking gently to precipitate DNA. The tube was held at an angle, the supernatant gently removed, and the DNA was air-dried. 100 or 150 µL TE pH 8.0 (including 10 mM Tris-HCl and 1mM EDTA) was added to the air-dried DNA, which was then dissolved at 70 °C for 3-4 hr.

PCR mix was prepared as follows: 1x Taq Buffer (Invitrogen, USA), 0.2 µM dNTP's, 0.2 µM Loxup2 primer (5'-TCCTGCACTTAGGTAAGATGCAAAGGC-3') (Fisher Scientific, PA, USA), 0.2 µM Screen1 primer (5'-GAACATGAACTGCAGCAGAGTGGT-3') (Fisher Scientific, PA, USA), 0.2 µM αlcwt primer (5'-GAACATGAACTGCAGCAGAGTGTA-3') (Fisher Scientific, PA, USA), 0.5 µL Taq polymerase (Invitrogen, USA) and 5 µL DNA in a final total volume of 30 µL. PCR samples were run as follows: first at 94 °C for 5 min, followed by 30 sec at 94 °C, 30 sec at 64 °C and 30 sec at 72 °C for 30 cycles and finally held at 4 °C.

DNA samples from the PCR reaction were prepared by adding 1x DNA loading buffer to each reaction tube, 20 µL of which was loaded into a 2.5% agarose gel and was electrophoresed at 134 mV for 30 min. DNA bands in the gel were photographed under UV light. WT yields a fragment of 390 bp, $Ca_v1.2DHP^{(-)}$ gives a fragment of 475 bp and $Ca_v1.2DHP^{(+)}$ has both fragments (Figure 4.1).

4.2.3 Slice preparation

The slice preparation was the same as that described in 3.2.1.

4.2.4 Nystatin-perforated patch clamp recording

This part was the same as that described in 2.2.2.

4.2.5 Drugs

This part was the same as that described in 2.2.2.

4.2.6 Data analysis

This part was the same as that described in 2.2.4 and 3.2.4.

4.3 Results

Only DA cells in the VTA identified according to criteria outlined in the Methods section were included.

4.3.1 DA cells of $Ca_v1.2DHP^{(-/-)}$ and WT mice have similar basic electrophysiological properties (Table 1)

In the WT group, most cells (15 of 27, 55.6%) were spontaneously active with single spike firing at a low basal firing frequency of 0.72±0.13 Hz; the remainder (12 of 27, 44.4%) was quiescent during baseline recording. Similarly, the majority of cells (27 of 44, 61.4%) in $Ca_v1.2DHP^{(-/-)}$ group showed pacemaker firing during baseline recording with a similar low frequency of 0.76±0.13 Hz (unpaired t test compared to that of WT group, $P>0.05$); 38.6% of cells (17 of 44) did not spontaneously fire. The resting membrane potentials (RMPs) were similar between the two groups (-53.94±1.07 mV for

WT mice vs. -52.76±1.13 mV for $Ca_v1.2DHP^{(-/-)}$ mice, unpaired t test, P>0.05). There was an apparent difference in RMPs of spiking and quiescent cells in both strains. In the WT mice, the RMP of spiking cells (-49.65±0.67 mV, n=15) was more depolarized than that of quiescent cells (-56.66±1.88 mV, n=12, unpaired t test, P<0.001). The same pattern was observed in $Ca_v1.2DHP^{(-/-)}$ mice (-50.67±0.91 mV for spiking cells, n=27, vs. -58.56±1.72 mV for quiescent cells, n=17, unpaired t test, P<0.001). There were no differences in RMPs of spiking cells (P>0.05) or quiescent cells (P>0.05) between wild type and congenic knock-in groups. In terms of spiking cells, there were no significant differences in spike amplitude (58.57±2.64 mV in WT mice vs. 57.75±2.17 mV in $Ca_v1.2DHP^{(-/-)}$ mice, unpaired t test, P>0.05), threshold (-38.91±0.84 mV in WT mice vs. -39.16±0.97 mV in $Ca_v1.2DHP^{(-/-)}$ mice, unpaired t test, P>0.05), half-width (2.79±0.14 ms in WT mice vs. 3.05±0.19 ms in $Ca_v1.2DHP^{(-/-)}$ mice, unpaired t test, P>0.05), or hyperpolarization after-potentials (-13.14±1.66 mV in WT mice vs. -13.77±1.32 mV in $Ca_v1.2DHP^{(-/-)}$, unpaired t test, P>0.05).

In addition, the average hyperpolarization following a brief application of 50 μM DA (within 90 sec) between the WT (-7.66±1.03 mV) and $Ca_v1.2DHP^{(-/-)}$ mice (-9.68±1.00 mV) was not significantly different (unpaired t test, P>0.05). When current injections were adjusted in each cell to result in a peak hyperpolarization of about −110 mV (Figure 1.5), the I_h sags between the WT mice (21.15±1.17 mV) and $Ca_v1.2DHP^{(-/-)}$ (19.29±0.92 mV) were not significantly different (unpaired t test, P>0.05).

4.3.2 The DHP site L-type calcium channel antagonist reduces single spike firing in both WT and $Ca_v1.2DHP^{(-/-)}$ mice

As mentioned in section 4.1, only $Ca_v1.2$ and $Ca_v1.3$ subtypes are expressed in VTA DA neurons, both of which are responsive to DHP site agonists and antagonists although $Ca_v1.3$ channels show a relative low responsiveness. As a result, it is difficult to identify the specific contribution of these L-type calcium channel subtypes to physiological functions. $Ca_v1.2DHP^{(-/-)}$ has a mutant DHP site on the $\alpha1$ subunit of $Ca_v1.2$ L-type calcium channels, which renders DHP modulators practically ineffective thereby providing a good tool to study the role of specific subtypes.

Bath application of 10 μM nifedipine, a DHP site L-type calcium channel antagonist, for 10 min in WT mice decreased baseline firing rate by 20.3±8.1% from 0.85±0.17 Hz to 0.70±0.21 Hz (n = 5, paired t test, $P<0.05$). Nifedipine is a lipophilic drug which is not easy to wash out, so the firing rate kept dropping by 39.1±6.3% to 0.58±0.18 Hz after washing out nifedipine for 7 min (paired t test, $P<0.05$) (Figure 4.2.1). Nifedipine blocks both $Ca_v1.2$ and $Ca_v1.3$ subtypes in the wild type, this result only underscores the importance of L-type calcium channels in basal firing without any clue as to the individual contributions from the two subtypes.

Determining which of the subtypes drives DA basal firing could be tested in $Ca_v1.2DHP^{(-/-)}$ mice because both are expressed in DA cells and the DHP site is not

functional in $Ca_v1.2DHP^{(-/-)}$ mice. Therefore, basal firing blocked by nifedipine represents the contribution from the $Ca_v1.3$ subtype. In $Ca_v1.2DHP^{(-/-)}$ mice, bath application of 10 μM nifedipine for 10 min reduced firing rates by 24.9±7.7% from 0.77±0.21 Hz to 0.63±0.20 Hz (n=5, paired t test, $P<0.05$) (Figure 4.2.2). A peak reduction of 34.0±9.2% to 0.57±0.18 Hz was recorded after washing out nifedipine for 7 min (paired t test, $P<0.05$). There was no significant difference in the reduction of basal firing by nifedipine between WT and $Ca_v1.2DHP^{(-/-)}$ mice (unpaired t test, $P>0.05$) (Figure 4.2.3), clearly indicating that the $Ca_v1.3$ subtype drives basal firing in VTA DA cells.

4.3.3 L-type calcium channel agonists convert single spiking to bursting in the mice

Burst firing is an important firing property of DA cells as detailed in Chapter 1. It has been shown that L-type calcium channel openers can induce firing pattern conversion in rats, so first of all, I would need to confirm that this happens in the WT mice as well before I could use the L-type transgenic strains. Bath application of 5 μM (S)-(-)-Bay K8644, a DHP site L-type calcium channel activator, for 7-11 min converted firing patterns from the quiescent state or single spiking to burst firing in 4 of 6 treated cells in WT mice (Figure 4.3.1), the same that I reported in rats. The percentage of converted burst firing in spontaneously firing cells (3 of 3) was much higher than that in quiescent cells (1 of 3).

In cells that were spontaneously firing, (S)-(-)-Bay K8644 first induced a membrane depolarization accompanied by an increase in firing rate, burst firing started after a varying period of latency. The lag between the start of drug application and burst firing ranged 9-33 min with an average time of 16 min. Within a burst firing cycle, action potentials fired with increasing frequencies followed by a pronounced post-burst hyperpolarization. The average intra-burst firing frequency was much higher than basal tonic frequency. The development of burst firing in quiescent cells was slightly different: they responded to (S)-(-)-Bay K8644 with a membrane depolarization followed by irregular single spiking that evolved into burst firing. The L-type calcium channel-mediated burst firing was long lasting even after prolonged washout (>30 min), however, it was readily inhibited by the L-type calcium channel blocker nifedipine (10 μM for 10 min, n=3, Figure 4.3.1). Density plots of ISIs in 2-second bins (Figure 4.3.2) showed an ISI distribution: the leftward shift of the main peak indicates higher firing frequencies and the second peak at much lower frequency represents the long pauses of firing between adjacent bursts.

Similarly, bath application of another L-channel opener FPL 64176 1-4 μM, a benzoylpyrole but not DHP site L-channel opener, for 8-24 min converted firing patterns from quiescent state or single spiking to burst firing in 7 of 11 treated cells (Figure 4.3.3), the same as in rats. Differently, however, application of 1 μM FPL 64176 did not induce any burst firing in 2 treated cells and 2 μM only induced bursting in 1 of 7 treated cells. The responses were dose-dependent: 4 μM FPL 64176 induced burst firing in 5 of 6 cells

that did not respond to 1 or 2 μM. And the percentage of burst firing in spontaneously firing cells (5 of 6) was much larger than that in quiescent cells (2 of 5). The time course was similar to that following (S)-(-)-Bay K8644, a depolarization and an increase in firing rates followed by a conversion of firing patterns 8-42 minutes (average: 25 min) after the start of application. The burst firing was long lasting, which could be transferred to single spike firing or totally blocked to no firing by the DHP site L-type calcium channel antagonist nifedipine (10 μM, 5-10 min, n=3).

4.3.4 The DHP site L-type calcium channel agonist converts firing patterns in $Ca_v1.2DHP^{(-/-)}$ mice

For identifying the role of different subtypes of L-channels in burst firing, $Ca_v1.2DHP^{(-/-)}$ mice were used, in which only $Ca_v1.3$ but not $Ca_v1.2$ L-type calcium channels are responsive to DHP modulators. Bath application of 5 μM (S)-(-)-Bay K8644 for 8-20 min induced burst firing in 4 of 9 cells tested in nine $Ca_v1.2DHP^{(-/-)}$ mice (Figure 4.4.1, 4.4.2), similar to what it did in WT mice. The time course of the response was also similar to that in WT mice, a depolarization and an increase in firing rates followed by a conversion of firing patterns, except that the lag between the start of drug application and burst firing was longer (range 27-40 min with the average time of 31 min). Percentage of converted burst firing in spontaneously firing cells (3 of 6) was also much higher than that in quiescent cells (1 of 3). Burst firing in 3 spontaneously firing cells was long lasting, the same as in WT mice, but only existed for 6 min in the quiescent cell.

Nifedipine (10 μM for 10 min), just as it did in WT mice, converted burst firing induced by the DHP site L-type calcium channel opener into single spike firing (n=3) (Figure 4.3.3).

4.3.5 DHP site L-type calcium channel antagonist inhibits burst firing induced by a non-DHP site L-type calcium channel agonist in $Ca_v1.2DHP^{(-/-)}$ mice

Similarly to what it did in WT mice, bath application of 2-4 μM FPL 64176 for 13-24 min also converted firing patterns from the quiescent state or single spiking to burst firing in 7 of 14 treated cells in $Ca_v1.2DHP^{(-/-)}$ mice (Figure 4.5). The responses were dose-dependent as well: 4 μM FPL 64176 induced burst firing in 3 of 6 cells that did not respond to 2 μM, and the percentage of burst firing in spontaneously firing cells (6 of 10) was much higher than that in quiescent cells (1 of 4). The time course was similar to that in WT mice, a depolarization and an increase in firing rates followed by a conversion of firing patterns 13-42 minutes (average: 21 min) after the start of application. The induced burst firing was long lasting except one in the quiescent cell which lasted for only 9 min. Nifedipine (10 μM for 5-13 min), a DHP site L-channel antagonist, also inhibited the burst firing: being converted back into single spike firing (n=4) or into protracted and slow bursting resembling irregular firing (n=2).

4.4 Discussion

The firing behavior of DA cells in the VTA is related to DA transmission associated with normal or abnormal expression of motivation and reward processing. L-type calcium channels play important roles in regulating VTA-DA cell firing, however, which subtype is involved is unclear. In this study, I used a transgenic mouse strain that lacks DHP functionality to differentiate L-type calcium channel subtypes in regulating burst firing of DA cells in the VTA. Our results indicate that $Ca_v1.3$ L-type calcium channels are crucial to regulating single spiking and burst firing in DA cells.

4.4.1 $Ca_v1.3$ L-type calcium channels play a major role in regulating firing properties

There are four subtypes of L-type calcium channels, although only $Ca_v1.2$ and $Ca_v1.3$ subtypes are expressed in DA cells (Chan, et al., 2007;Takada, et al., 2001). L-type calcium channels are often assumed to be high voltage-activated (e.g. activate between -40~-20 mV membrane potentials) (Ertel, et al., 2000) and $Ca_v1.2$ L-type calcium channels display this typical voltage dependency (Mori, et al., 1993). However, $Ca_v1.3$ L-type calcium channels activate at relatively hyperpolarized membrane potentials (between -60~-40 mV) and are incompletely inhibited by DHP antagonists (Xu and Lipscombe, 2001). The large difference in activation thresholds between these two subtypes implies that they may be coupled to different molecular signaling pathways

(Zhang, et al., 2006) so as to mediate different physiological roles in neuronal electrical tasks. For example, $Ca_v1.3$ L-type calcium channels activate at relatively hyperpolarized membrane potentials (around -50 or -55 mV) (Xu and Lipscombe, 2001), which is near resting membrane potential, so $Ca_v1.3$ channels are likely to be important in mediating Ca^{2+} influx in response to relatively small membrane depolarization. Such properties may be important for sustaining spontaneous rhythmic firing in neurons, traditionally attributed to the activities of low-threshold T-type Ca^{2+} channels (Bean, 1989;Ertel, et al., 2000). Consistent with this, the DHP antagonist nimodipine partially suppresses spontaneous intracellular calcium oscillations and slows rhythmic firing in postnatal cerebellar Purkinje cells (Liljelund, et al., 2000). More direct evidence for the involvement of $Ca_v1.3$ channels in driving rhythmic activities in excitable cells comes from a study of $Ca_v1.3$-deficient mice whose phenotype includes compromised sinoatrial node function (Platzer, et al., 2000). In line with this, our study shows that $Ca_v1.3$ channels mediated rhythmic firing in DA neurons in the VTA because compared with wild type mice, the DHP antagonist nifedipine slowed spontaneous rhythmic firing to a similar extent in $Ca_v1.2$ knock-in mice, in which $Ca_v1.2$ channels were not sensitive to DHP antagonists and only $Ca_v1.3$ channels were inhibited by the DHP antagonist nifedipine. It is consistent with the finding that $Ca_v1.3$ channels mediate the spontaneous firing activities of DA neurons in the substantia nigra of adult mice (Chan, et al., 2007).

Different from rhythmic firing, burst firing is a combination of subthreshold depolarization and clustered action potential firing, which is likely to be mediated by both

$Ca_v1.3$ (activated at relatively hyperpolarized membrane potentials and assumed to mediate the underlying subthreshold depolarization) (Xu and Lipscombe, 2001) and $Ca_v1.2$ (high-voltage activated and hypothesized to be involved in the clustered fast firing) (Mori, et al., 1993). It is reasonable to presume that $Ca_v1.3$ L-type calcium channels mediate the subthreshold depolarization that raises the membrane potential to activate sodium channels initiating firing and then $Ca_v1.2$ L-type calcium channels take over to maintain sequential firing. Calcium influx precipitates a yet unknown calcium-dependent process to generate a post-burst hyperpolarization which completes a bursting cycle. However, my results support the hypothesis that only $Ca_v1.3$ L-type calcium channels are involved in bursting because: 1) activating both $Ca_v1.2$ and $Ca_v1.3$ subtypes ((S)-(-)-Bay K8644 in wild type slices) induced burst firing similarly to activating $Ca_v1.3$ subtype alone ((S)-(-)-Bay K8644 in $Ca_v1.2DHP^{(-/-)}$ slices); 2) Bay K-induced burst firing could be blocked by nifedipine in both wild type slices (effective for both subtypes) and $Ca_v1.2DHP^{(-/-)}$ slices (effective only for $Ca_v1.3$); 3) activating both subtypes with FPL 64176 induced similar burst firing which could be blocked to the same extent with nifedipine (which blocks $Ca_v1.2$ and $Ca_v1.3$ in wild type slices and $Ca_v1.3$ only in $Ca_v1.2DHP^{(-/-)}$ slices). These results collectively demonstrate that $Ca_v1.3$ L-type calcium channels are obligatory for burst firing. $Ca_v1.2$ L-type calcium channels are not necessary for burst firing but might aid it in some way because our data showed that the latency to bursting (lag time) was slightly longer in $Ca_v1.2DHP^{(-/-)}$ slices than in WT ones, and in a few cases induced bursting did not last as long. The mechanism for this might be related to contributions to the hump potential, which is already shown to be

mediated by L-type calcium channels (Zhang, et al., 2005), similar to the plateau potential underlying bi-stable membrane behavior in motorneurons in which $Ca_v1.3$ L-type calcium channels are involved (Carlin, et al., 2000;Hsiao, et al., 1998).

4.4.2 $Ca_v1.3$ L-type calcium channels are functionally important

$Ca_v1.3$ L-type calcium channels are one of the four subtypes of L-type calcium channels, but due to technical difficulties in pharmacologically isolating $Ca_v1.3$ from $Ca_v1.2$ L-type calcium channels, both of which are abundantly expressed in neurons, the specific functional roles of $Ca_v1.3$ are still obscure. With the development of transgenic animals, $Ca_v1.3$ L-type calcium channels have been found to be implicated in addiction and other diseases. For example, $Ca_v1.3$-deficient mice lack amphetamine-sensitized locomotor activity (Giordano, et al., 2006) and exhibit an antidepressant-like phenotype (Striessnig, et al., 2006). In addition, the expression of $Ca_v1.3$ L-type calcium channels is decreased by chronic morphine treatment in midbrain regions of mice (Haller, et al., 2008). More importantly, $Ca_v1.3$ L-channels can achieve all these behaviors by its role in regulating pacemaker and burst firing as reported here because fast pacemaker and burst firing elevate terminal DA levels more efficiently and all of these behaviors involve increased DA transmission. $Ca_v1.3$ L-type calcium channels play a crucial role in maintaining rhythmic pacemaking firing in adult substantial nigra neurons, and blocking $Ca_v1.3$ L-type calcium channels in adult neurons reverts them to a juvenile form of pacemaking firing dependent on Na^+ channels and protects these neurons in a model of

Parkinson's disease (Chan, et al., 2007). Moreover, addiction is a learned behavior and $Ca_V1.3$ L-channels are also shown to facilitate learning and memory such as in mediating the consolidation of contextually conditioned fear in mice (McKinney and Murphy, 2006). Taken together, $Ca_V1.3$ L-type calcium channels might play a significant role in central DA transmission and its related pathologies such as drug abuse. Further experiments on the role of L-type calcium channel subtypes are now possible through the use of these L-type specific transgenic mouse strains.

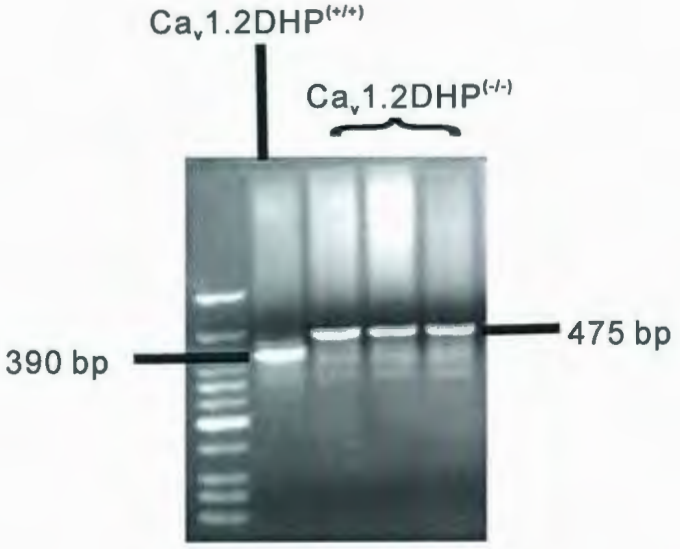

Figure 4.1.Genotyping PCRs show that $Ca_v1.2DHP^{(+/+)}$ mice yield a band of 390 bp and $Ca_v1.2DHP^{(-/-)}$ has a band of 475 bp.

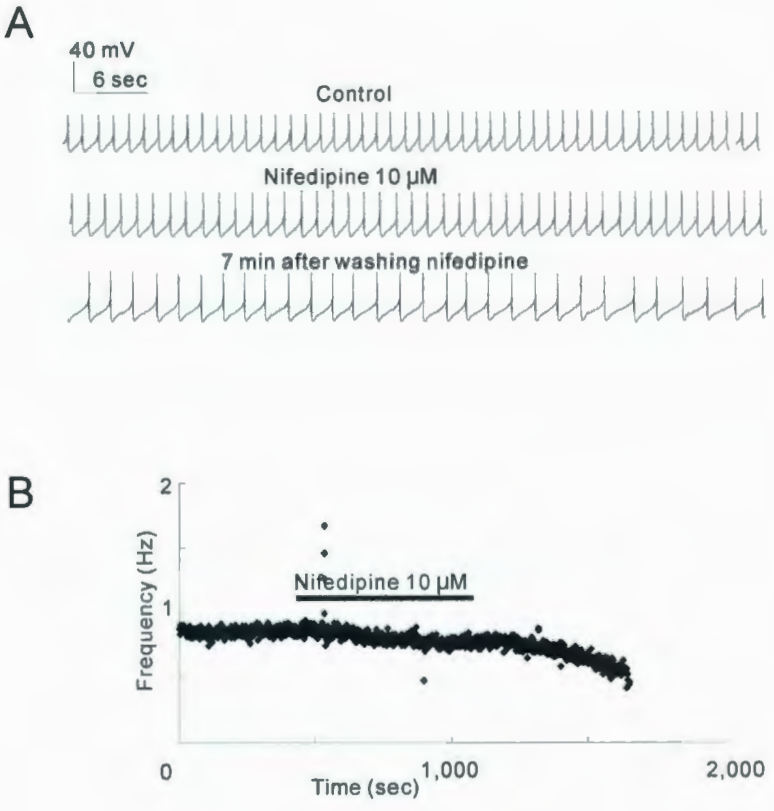

Figure 4.2.1 The DHP site L-type calcium channel antagonist nifedipine decreases basal firing rate in $Ca_v1.2DHP^{(+/+)}$ mice. A. Current clamp recording from a representative cell showing firing rates in control, nifedipine (10 µM for 10 min) and after washing out nifedipine for 7 min. **B.** Changes in instantaneous firing frequencies following nifedipine from the same cells as in A.

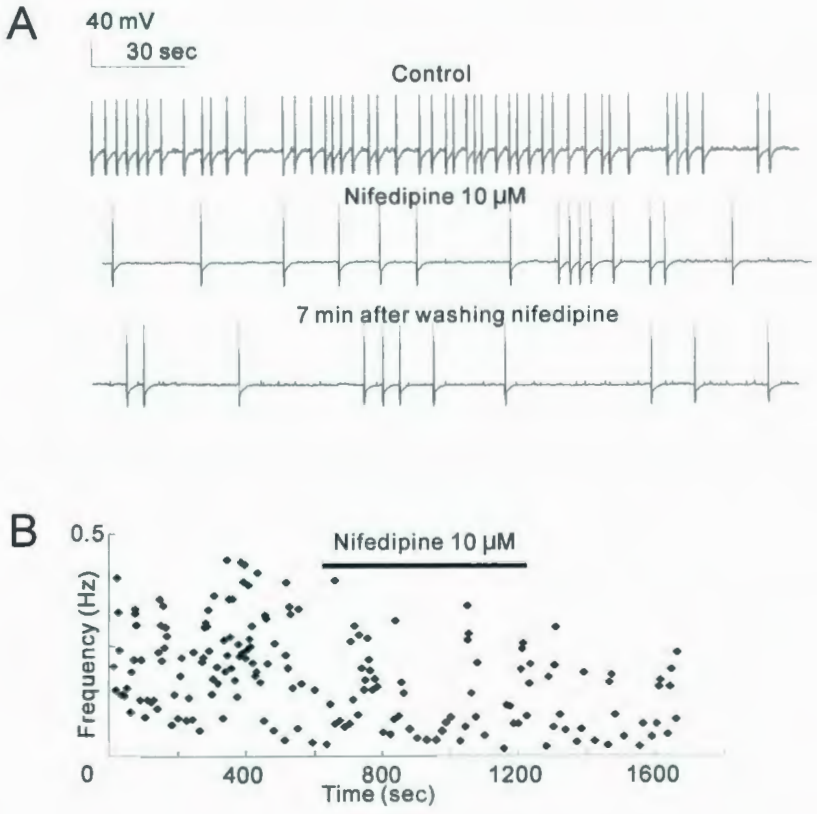

Figure 4.2.2 The DHP site L-type calcium channel antagonist nifedipine decreases basal firing rate in $Ca_v1.2DHP^{(-/-)}$ mice. A. Current clamp recording from a representative cell showing firing rates in control, nifedipine (10 µM for 10 min) and after washing out nifedipine for 7 min. **B.** Changes in instantaneous firing frequencies following nifedipine from the same cells as in A.

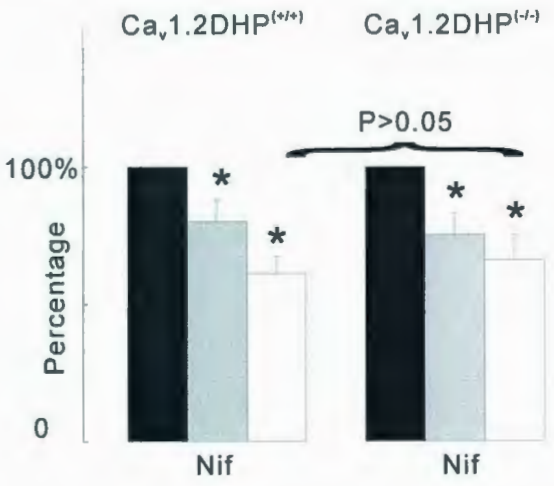

Figure 4.2.3 Percentage changes in firing rate reductions following nifedipine (10μM for 10 min, gray) and 7 min after washing out nifedipine (white) compared with control (black) in Ca$_v$1.2DHP$^{(+/+)}$ and Ca$_v$1.2DHP$^{(-/-)}$ mice. (* means P<0.05)

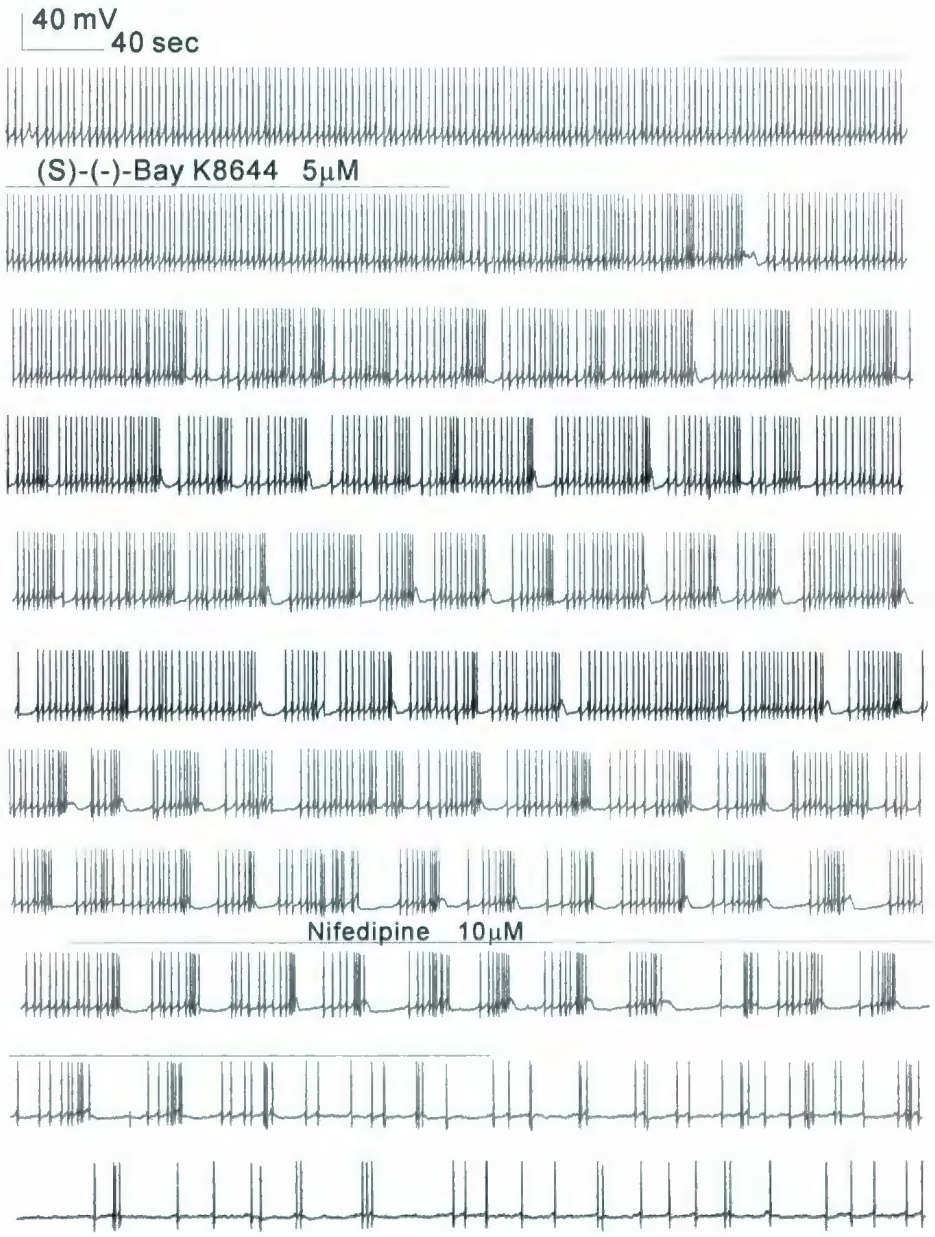

Figure 4.3.1 Continuous current clamp recording from a representative cell showing that (S)-(-)-Bay K8644 (5 μM) converted regular firing to burst firing which could be blocked by nifedipine (10 μM) in $Ca_v1.2DHP^{(+/+)}$ mice.

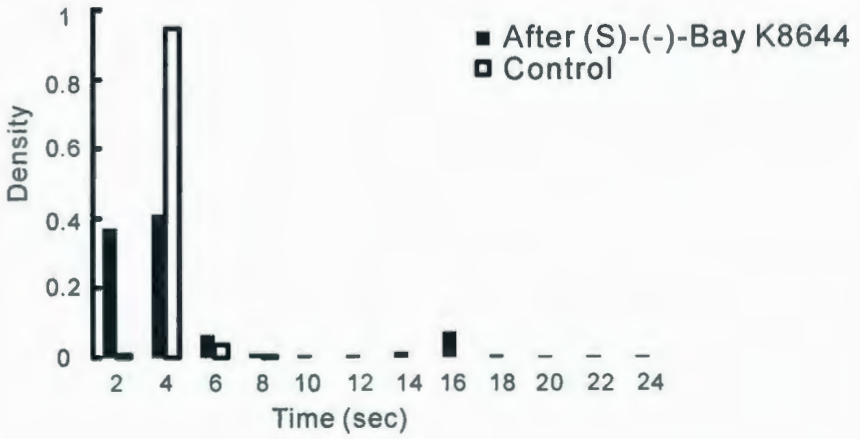

Figure 4.3.2 Density plot of interspike intervals (ISI) in 2-second bins in control conditions (210 events) and following (S)-(-)-Bay K8644 application (547 events) in $Ca_v1.2DHP^{(+/+)}$ mice.
(S)-(-)-Bay K8644 dramatically shifted the peak to the left and gave rise to a secondary peak corresponding to the frequency of burst firing cycles.

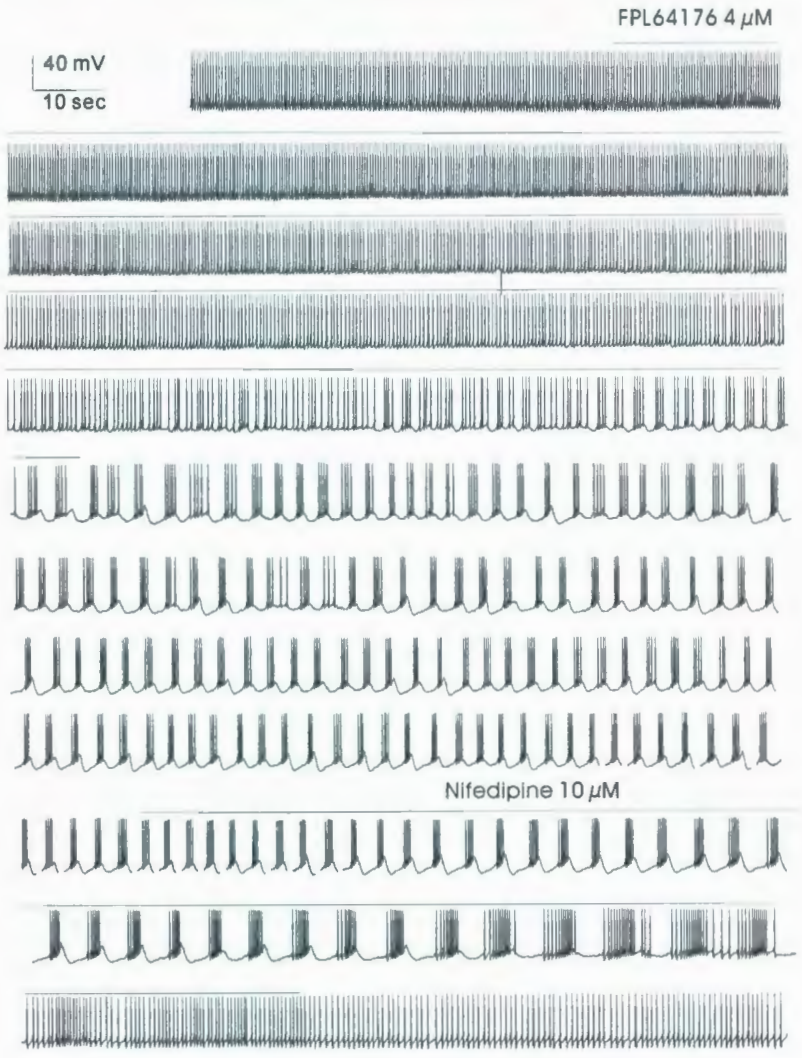

Figure 4.3.3 Continuous current clamp recording from a representative cell showing that FPL 64176 (4 μM) converted regular firing to burst firing which could be blocked by nifedipine (10 μM) in $Ca_v1.2DHP^{(+/+)}$ mice.

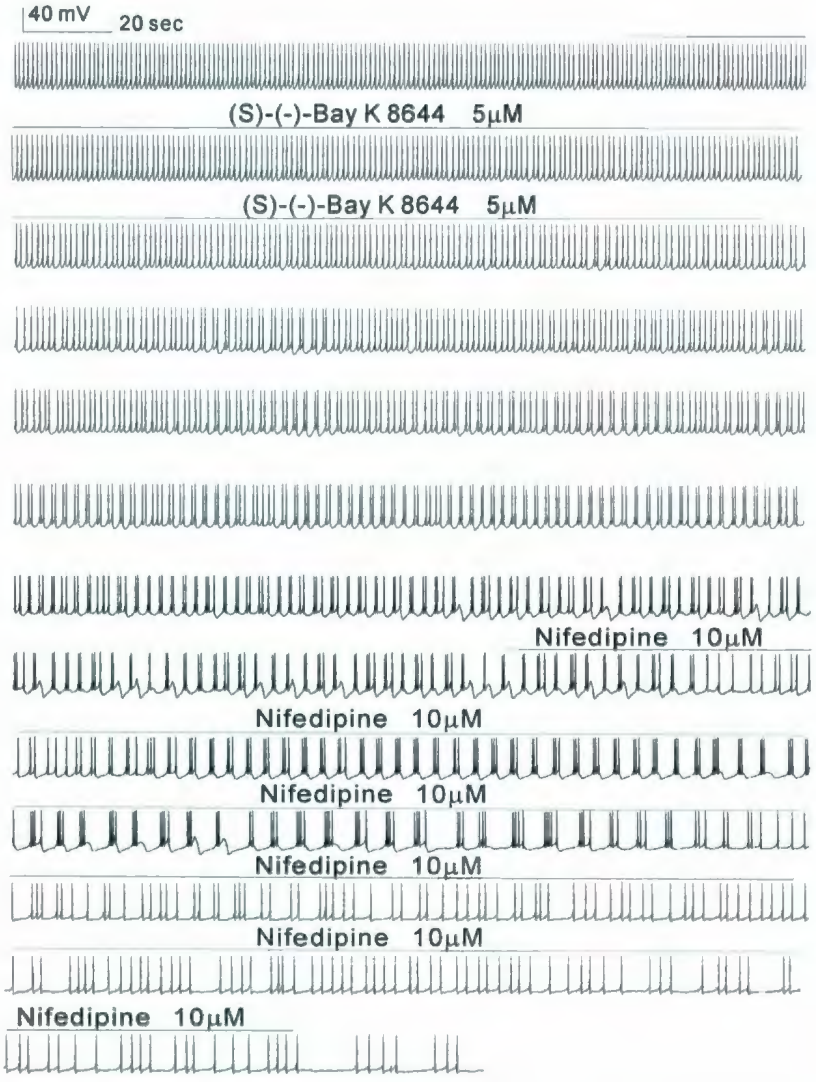

Figure 4.4.1 Continuous current clamp recording from a representative cell showing that (S)-(-)-Bay K8644 (5 μM) converted regular firing to burst firing which could be blocked by nifedipine (10 μM) in $Ca_v1.2DHP^{(-/-)}$ mice.

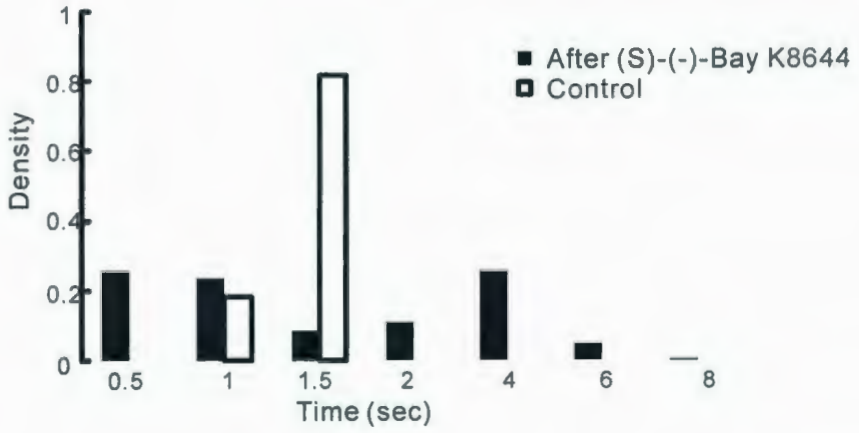

Fig 4.4.2 Density plot of interspike intervals (ISI) in 0.5- (<2 sec) or 2-second (>2 sec) bins in control conditions (859 events) and following (S)-(-)-Bay K8644 application (585 events) in $Ca_v1.2DHP^{(-/-)}$ mice. (S)-(-)-Bay K8644 dramatically shifted the primary peak to the left and gave rise to a secondary peak corresponding to the frequency of burst firing cycles.

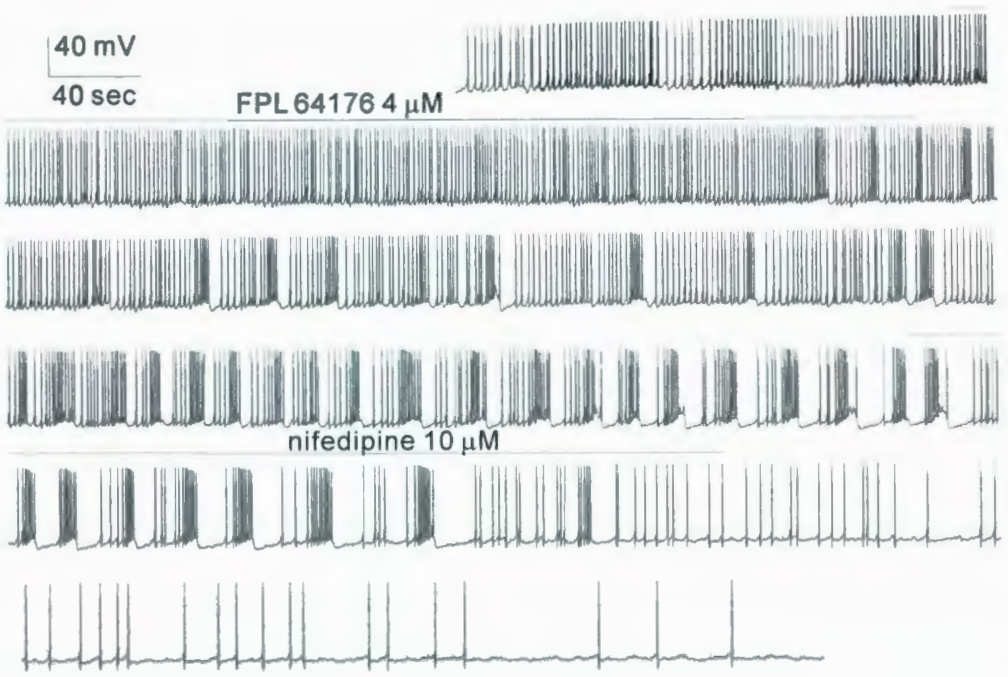

Figure 4.5 Continuous current clamp recording from a representative cell showing that FPL 64176 (4 μM) converted regular firing to burst firing which could be blocked by nifedipine (10 μM) in $Ca_v1.2DHP^{(-/-)}$ mice.

Table 1

the basic electrophysiological properties in dopaminergic cells between $Ca_v1.2DHP^{(+/+)}$ and $Ca_v1.2DHP^{(-/-)}$ mice

	$Ca_v1.2DHP^{(+/+)}$(WT)	$Ca_v1.2DHP^{(-/-)}$	*P*
number of spontaneously active cells	15 of 27, 55.6%	27 of 44, 61.4%	
basal firing frequencies (Hz)	0.72±0.13	0.76±0.13	>0.05
resting membrane pottials (mV)	-53.94±1.07	-52.76±1.13	>0.05
spiking amplitude (mV)	58.57±2.64	57.75±2.17	>0.05
spiking threshold (mV)	-38.91±0.84	-39.16±0.97	>0.05
spiking half-width (ms)	2.79±0.14	3.05±0.19	>0.05
hyperpolarization after-potentials (mV)	-13.14±1.66	-13.77±1.32	>0.05
hyperpolarization following an application of 50 μM dopamine (mV)	-7.66±1.03	-9.68±1.00	>0.05
I_h sag (mV)	21.15±1.17	19.29±0.92	>0.05

Chapter 5 Summary

5.1 Technical considerations

Work presented in this thesis was designed to study the electrophysiological properties of DA neurons, especially their firing activities including bursting and pacemaker single spiking. Neuronal firing in slice preparations can be affected by a number of technical factors, such as the age of animals and the temperature at which all patch clamp recordings were made. This part summarizes the technical factors related to my thesis work.

5.1.1 Age of animals and DA firing activity

The age of animals has been considered as an important factor involving molecular signals and electrical activities. During development, the DA system undergoes important changes. For example, DA innervations to the forebrain and DA receptors in the NAc and striatum increase rapidly from birth to reach a peak around 40 days old in rodents, and decrease gradually thereafter (Andersen, et al., 1997;Tarazi and Baldessarini, 2000). Similarly, DA levels in the striatum also increase from birth to adolescence in rodents

(Andersen and Gazzara, 1996;Laviola, et al., 2001;Teicher, et al., 1993). Concerning L-type calcium channels, age effects exist as well. For instance, after the second postnatal week in mice, the contribution of $Ca_v1.3$ L-channels to somatodendritic L-channel currents increases and at P28, the percentage of $Ca_v1.3$ L-channel currents increases by about 10% compared with that at P14-17 (Chan, et al., 2007). There is no reference indicating at what age these developing channels stabilized. Whereas currents attributable to $Ca_v1.2$ L-channel remain largely stable (Chan, et al., 2007). Given that this thesis work is mostly concerned with the firing behavior of DA cells in acute brain slices, it would be important to explore whether these age-related changes in the development of the central DA system as well as in the expression of L-type calcium channels have any significant influence on DA firing.

DA neurons have been shown in an *in vivo* study to display progressive increases in single spiking rates and in burst firing occurrence from birth to early adolescence (28-35 days old) (Pitts, et al., 1990;Tepper, et al., 1990), when autoreceptors in the midbrain appear to be functionally mature (Wang and Pitts, 1995). Firing activities are higher during mid adolescence (35-42 days old) and decline progressively during adulthood (75-90 days old) (Freeman, et al., 1989;Lavin and Drucker-Colin, 1991). As introduced in Chapter 1, DA neurons in acute slice preparations have very different firing patterns in that they barely display burst firing spontaneously even with chemical stimulations. However, Mereu reported that DA neurons in brain slices from immature rats (15-21 days

old) exhibited not only pacemaker firing but also irregular and burst firing pattern (28.3 and 18.3% respectively) (Mereu, et al., 1997), an effect that is attributable to NMDA receptor activation as they stop bursting when NMDA receptor sensitivity declines with age. NMDA does induce burst firing in adult animals, although a hyperpolarizing current is needed for this induction (Overton and Clark, 1997). In my view, the age of the animal does not confound the results in this thesis primarily because animals used in a given set of experiments did not span major developmental stages (9-21 days old). As a result, L-type calcium channel-induced burst firing used as a test model in rats is quite stable and predictable, 80.3% of DA neurons in brain slices harvested from pre-weaning rats displayed a burst firing pattern following L-type calcium channel activation. I also tested this model in adult mice (> 2 month old) presented in Chapter 4 to specifically examine the L-type calcium channel subtypes in transgenic mice, 63.6% of DA neurons could be induced to burst fire. Age does not appear to have a significant role in the expression of burst firing specifically induced in DA cells from acute brain slices. However, the percentage of induced bursting is higher in juvenile rats than in mature mice, but whether this is related to age is not known. Since mice (39°C) have higher body temperatures than rats (37°C), the difference between body temperature and our recording temperature may also contribute to this.

5.1.2 The effects of temperature on neuronal firing

All patch clamp recordings in this thesis work were conducted at room temperature (22°C). Temperature regulates the rate of enzyme-catalyzed reactions and has effects on the function of ion channel proteins. For instance, I_h is found to be sensitive to temperature being more conductive at higher temperatures (Vargas and Lucero, 1999). In addition, L-type calcium channels display increased conductance, more hyperpolarized activation voltages and accelerated recovery from inactivation over a given time at higher temperatures of 36-37°C compared to room temperature at 21-23°C (Huneke, et al., 2004;Peloquin, et al., 2008). Therefore, it is certain that I_h and L-type calcium channels are less efficient at room temperature, which may be reflected in the fact that the basal firing frequency of DA neurons in our experiments (0.41±0.04 Hz in rats) is lower than that reported at higher temperatures (spontaneous spike frequency is >1 Hz) (Johnson and North, 1992b;Lacey, et al., 1989). It is also possible that temperature contributes to the discrepancy in induced bursting rates in rats and mice because mice (39°C) have higher body temperature than rats (37°C) giving rise to a bigger differential temperature at 22°C at which the recording was conducted. This may account for the lower percentage of burst firing following the application of L-type calcium channel activators in mice (63.6%) relative to that in rats (80.3%).

5.1.3 Recording methods used in my thesis work

The perforated patch clamp recording method was used in almost all experiments with only two exceptions: dialysis of PKM into the recorded cell and isolation of Ca^{2+} currents, both of which required conventional whole-cell patch clamp recording for intracellular delivery of the chemical tools. Perforated patch clamp recordings have some advantages over conventional whole-cell recordings: the channels formed by nystatin used in my perforated recordings are smaller and are impermeable to molecules larger than glucose, which means that only small ions such as Na^+, K^+, Cl^- can pass through while Ca^{2+} and second messengers can not. Therefore, recordings can be conducted without dialyzing important substances from the cell's cytoplasm. Currents run down significantly more slowly and second-messenger cascades and mechanisms important to cell signaling and channel regulation are kept operative (Fu, et al., 2003). The reason that I observed L-type calcium channel bursting could be due to the perforated recording I used in these experiments. As detailed in previous chapters, L-type calcium channel-induced bursting was dependent on Ca^{2+} and intracellular signaling molecules such as PKM, which are easily dialyzed in conventional whole-cell recording but remain perfectly intact in perforated recording.

5.1.4 Animal species used in my thesis work

It has been shown that there are differences in anatomy and physiological functions among different animal species. For example, the total number of TH-positive (TH, tyrosine hydroxylase) cells and the expansion of their innervation territory, especially in the neocortex, are much less in rodents than in monkeys and humans (Chu, et al., 2002;Emborg, et al., 1998;German and Manaye, 1993;Lewis, et al., 1998;Nelson, et al., 1996). As described in previous chapters, animal species used in my thesis work are rats and mice, which don't show many differences in anatomy and functions. In terms of L-type calcium channel-induced bursting, there was almost no qualitative difference in bursting induction and the characteristics of bursting itself except a small difference in drug concentration and lag time between the start of drug application and the onset of bursting. Therefore, both of the species can be used in the study of L-type calcium channel-induced bursting. However, mice were used in my later projects because there are transgenic models available to further explore the mechanism of L-type calcium channel-induced bursting. As indicated in previous chapters, the L-type calcium channel and PKC are two important signaling steps involved in the regulation of firing activities but there are no available pharmacological drugs to selectively activate or inhibit the specific subtypes of L-type calcium channels or PKC isoforms to isolate or identify their specific roles. Transgenic mouse models aid in this line of exploration. For instance, two mouse models are available to dissect the physiological role of $Ca_v1.2$ and $Ca_v1.3$ subtypes of L-type calcium channels: in $Ca_v1.3^{(-/-)}$ mice, the $Ca_v1.3$ α1-gene is knocked

out thereby $Ca_v1.3$-dependent cellular functions and $Ca_v1.3$-mediated DHP binding are lost (Platzer, et al., 2000); in $Ca_v1.2DHP^{(-/-)}$ mice, which were used in my thesis work, $Ca_v1.2$ $\alpha1$-gene is point-mutated thereby $Ca_v1.2$ channels are normally expressed but $Ca_v1.2$-mediated DHP binding and effects are lost, thus, DHP modulators are $Ca_v1.3$-selective in these animals (Sinnegger-Brauns, et al., 2004). Similarly, individual PKC isoform knockouts generated over the past few years provide the possibility of further investigating PKC isoforms in the regulation of DA firing activities. Cross breeding of PKC and L-type calcium channel transgenic mice can produce double knockouts and provide defining information on the relationship between PKC isoforms and L-type calcium channel subtypes in neuronal firing activities, which will be interesting projects in the future.

5.1.5 Criteria for identifying DA neurons used in my thesis work

The criteria for identifying DA neurons in my thesis work were indirect electrophysiological characterizations: a prominent I_h and an apparent DA-induced hyperpolarization, which are commonly used by investigators because both can be easily obtained during perforated patch clamp recordings without any other effects on neurons' activities (Johnson, et al., 1992;Lacey, et al., 1989;Zhang, et al., 2005). This has been used in many reports, especially earlier ones, as a reliable electrophysiological fingerprinting. However, recent evidence suggests that it is not as reliable as earlier claims because these general electrophysiological properties used for identifying DA

neurons, including I_h and DA-induced hyperpolarization, are found in a large proportion of TH-negative VTA neurons. Moreover, some TH-positive VTA neurons don't display these typical electrophysiological properties (Margolis, et al., 2006). For example, Lammel et al (Lammel, et al., 2008) showed that the TH-positive mesocortical VTA neurons do not have DA-induced inhibition and I_h. It implies that there were some non-DA neurons in the putative DA neurons recorded in my thesis work. The more accurate method is neurochemical identification of TH in VTA neurons. Therefore, loading biocytin into the pipette and breaking in to let biocytin flow into the cell after finishing perforated patch recordings would allow post-hoc identification of DA neurons accurately with double fluorescence immunocytochemistry for TH and biocytin. An alterative method is the use of TH-EGFP transgenic mice (Jomphe, et al., 2005), in which midbrain DA neurons can be directly visualized due to the expression of the enhanced green fluorescent protein (EGFP) under the control of the TH promoter.

5.2 Summary of main findings

5.2.1 The L-type calcium channel-induced burst firing as an *in vitro* model

One fundamental question of my thesis work is to identify cellular mechanisms regulating burst firing in DA neurons. Compared to *in vivo* studies, *in vitro* studies provide ways to manipulate specific steps of the signaling pathways involved. However, spontaneous (also called natural) burst firing barely exists in *in vitro* brain slices

(Grenhoff, et al., 1988). It has been reported that some neurotransmitters or their analogs such as carbachol or NMDA are capable of inducing burst firing in acute brain slices, but there are technical problems associated with these *in vitro* burst firing models. For example, the percentage of burst firing induced by carbachol is very low (<20%) which limits its use as an effective model (Zhang, et al., 2005). The proportion of NMDA-induced bursting is higher (around 40-50%) but it needs a hyperpolarizing current to induce bursting. It has been debated whether NMDA-induced bursting is relevant to bursting that occurs in behaving animals (Overton and Clark, 1997). The major point in this debate is that NMDA-induced bursts are sodium-dependent by not calcium-dependent, whereas natural bursts in DA neurons are critically dependent on intracellular calcium. Also, there are technical difficulties in studying the Ca^{2+} dependency of NMDA-induced bursting in that divalent cations used as Ca^{2+} channel blockers, such as NiCl and others, react with NMDA in solution making it difficult to know the effective dose of the ligand as well as the blockers (Gavazzo, et al., 2006;Fayyazuddin, et al., 2000). By contrast, L-type calcium channel-induced bursting is a simple and effective model to further explore the underlying signaling pathways, especially the role of Ca^{2+}, at the cellular level.

Firstly, L-type calcium channel-induced bursting is very reliable: it can be induced in a high percentage of cells tested in different animal species. For example, the L-type calcium channel opener FPL 64176 at a commonly used concentration (1 μM) could

induce bursting in 63.5% of putative DA cells in rats, and at a higher concentration (4 μM) this percentage could reach 80.3%. For mice, 4 μM could induce bursting in 63.6% of cells at room temperature. This is very important for a model to be used in further studies. Secondly, the properties of L-type calcium channel-induced bursting are similar to natural bursting. Just as described in sections 2.3 and 4.3, within a bursting cycle induced by L-type calcium channel activation, action potentials were fired with increasing frequencies followed by a pronounced post-burst hyperpolarization, a pattern that fits well with natural bursting. Thirdly, there are established chemicals to manipulate the L-type calcium channel and there are no adverse reactions with common modulators of other Ca^{2+} channels. Bath application of L-type calcium channel openers alone is sufficient for burst induction, and no other interventions are needed for burst induction or maintenance.

The subtype of the L-type calcium channel involved in this model is restricted to only $Ca_v1.2$ and $Ca_v1.3$ since they are the only subtypes expressed in DA neurons. In addition, L-type calcium channels are sensitive to DHP modulators therefore it is easy to rule out the involvement of other Ca^{2+} channels. Moreover, L-type calcium channel transgenic mouse strains exist to study the role of individual subtypes (Platzer, et al., 2000;Sinnegger-Brauns, et al., 2004), such as the $Ca_v1.2DHP^{(-/-)}$ strain. $Ca_v1.3$ L-type calcium channels are ideally positioned to regulate firing modes because they have a low activation potential threshold (Durante, et al., 2004;Xu and Lipscombe, 2001) and it has been found that pacemaking and burst firing are Ca^{2+}-dependent. I verified this

hypothesis and found that $Ca_V1.3$ L-type calcium channels mediated regular firing and played a crucial role in burst firing. In mice whose $Ca_V1.2$ L-type calcium channels have a dramatically reduced sensitivity to DHP, the DHP site L-type calcium channel opener (S)-(-)-Bay K8644 induced burst firing. Similarly, firing induced by a non-DHP site L-type calcium channel opener could be blocked by the DHP site L-channel inhibitor nifedipine. All of these results strongly suggest a dominant role for $Ca_V1.3$ in mediating burst firing of DA neurons in brain slices.

To establish L-type calcium channel-induced bursting as a valid *in intro* model, one would need to examine functional correlations of the induced burst firing and DA-related pathologies. While the notion that L-type calcium channel-induced bursting as a basis of enhanced DA transmission in diseases is yet to be explored, it is known, however, that L-type calcium channels are implicated in addiction and other diseases. For example, repeated amphetamine injections increase the expression of α1C L-type calcium channel subunits in the VTA (Rajadhyaksha, et al., 2004) and repeated stimulation of L-type calcium channels in the VTA mimics the initiation of behavioral sensitization to cocaine (Licata, et al., 2000), while direct injection of L-type calcium channel antagonists into the VTA attenuates the development of psychostimulant-induced behavioral sensitization (Licata, et al., 2004). L-type calcium channels can mediate all of these behaviors by their role in regulating the pacemaker and burst firing reported in my thesis work because burst firing elevates terminal field DA levels more efficiently and all of these behaviors arise

from increased DA transmission. Therefore, this model can serve as an electrophysiological testing platform to screen cellular signaling pathways which underlie affective behaviors such as addiction. In summary, the aforementioned arguments demonstrate that L-type calcium channel-induced bursting is a simple effective bursting model *in vitro* which has characteristics of natural bursting.

It is noteworthy that a feature of this model is that a lag exists between the start of drug application and the appearance of burst firing, which calls for stable recordings for a relatively long period of time. For instance, in my hands, the lag ranged from 5-25 min with an average of 13 min following a 5-min application of FPL 64176 in rats and 8-42 min with an average of 25 min following 8-24 min application of FPL 64176 in C57BL/6 mice. This means that the cell being recorded needs to be stable for at least 1 hour for a full expression of burst firing. Modifications to the procedure such as increasing the concentration of FPL 64176 or warming up the slices to 35°C, may speed up the onset of burst firing. However, it is highly unlikely that this lag is due to randomness because L-type calcium channel openers cause increased firing and depolarization within a few minutes, it is only that burst firing takes much longer to be induced. It could be that in different cells L-type calcium channel couplings are slightly different and the resulting signaling cascade takes different amounts of time to develop, or the temperature used for slice perfusion is not optimal for PKC-PKM enzymatic conversion.

5.2.2 PKC (PKM) signaling pathway in regulation of DA firing activities

The key to L-type calcium channel-induced burst firing is Ca^{2+} influx. While there are many routes of Ca^{2+} entry into the cell, many of which have specific couplings in DA neurons, it is not known what signaling pathway is coupled to Ca^{2+} influx through L-type calcium channels. The exploration of this issue has led to the discovery of the PKC (PKM) signaling pathway in the regulation of firing activities of putative DA neurons of the VTA.

PKC includes three classes: conventional, atypical and novel PKCs, many of which are shown to be expressed in DA neurons (Yoshihara, et al., 1991). PKC can phosphorylate many different substrates including L-type calcium channels at multiple serine/threonine sites. For example, it has been shown that phosphorylation of the $Ca_v1.2$ by PKC leads to positive modulation of the channel (Yang, et al., 2005) so that it becomes easier to open and conducts more Ca^{2+} ions. In addition, positive modulation of L-type calcium channels by PKC has also been reported in native tissues. For example, M_2 mAChR stimulation requires PKC to increase $Ca_v1.2$ currents in rabbit portal vein myocytes (Callaghan, et al., 2004) and M_1 receptor activation involves PKC to mediate facilitation of L-type calcium channels in dissociated autonomic neurons from the major pelvic ganglion (Sculptoreanu, et al., 2001). In our hands, PKC inhibitors reduced

carbachol-induced increases in nifedipine-sensitive Ca^{2+} currents, clearly showing that PKC regulates L-type calcium channels to affect firing activities of midbrain DA neurons. PKC has been shown to play a role in drug abuse. For instance, repeated administration of cocaine increases overall PKC activity in the VTA (Steketee, et al., 1998), while injection of PKC inhibitors into the VTA reduces cocaine-induced DA release in the NAc (Steketee, 1993) and delays the onset of behavioral sensitization (Steketee, 1994;1997). The PKC signaling pathway regulating firing activities of DA neurons might underlie drug abuse, an idea that is worth further exploration in the future.

PKM is a persistently active catalytic subunit of PKC. Elevated intracellular Ca^{2+} has been shown to activate calpain (Goll, et al., 2003), a protease that cleaves the PKC catalytic domain from the regulatory domain (Al and Cohen, 1993;Cressman, et al., 1995;Kishimoto, et al., 1983) to generate PKM. Since its discovery in the 1970s (Inoue, et al., 1977), its function remained obscure until recent reports associating it with learning and memory (Osten, et al., 1996;Sacktor, et al., 1993). This thesis work reports for the first time that PKM is involved in regulating DA firing activities.

It is reasonable to propose that PKM, the persistently active catalytic subunit of PKC, is a critical step in L-type calcium channel-induced burst firing. The evidence is as follows (Figure 2.8): (1) when burst firing was induced by L-type calcium channel

activation, chelerythrine, a PKC catalytic domain inhibitor, reversibly blocked burst firing, whereas calphostin C, a regulatory domain inhibitor did not; (2) L-type calcium channel openers induced burst firing accompanied by increased levels of phosphorylated PKM; (3) inhibition of the Ca^{2+}-dependent protease calpain reduced the level of phosphorylated PKM and concomitantly blocked burst firing; (4) depletion of PKC isoforms following prolonged incubation with PMA prevented L-type calcium channel-induced burst firing; (5) direct loading of the cell with purified PKM through the recording pipette induced burst firing in a similar manner to Ca^{2+} influx. Two conclusions are apparent from the collective evidence: (a) PKM is a critical coupling to L-type calcium channels; (b) PKM is required in burst firing. The need for PKM generation to induce burst firing also helps explain why it takes time for bursting to develop following L-type calcium channel opening. In addition, the persistent active PKM may sustain an enhanced activity of a signaling loop that keeps DA cells in bursting mode. One scenario is that Ca^{2+} influx through the L-type calcium channel activates a Ca^{2+}-dependent protease cleaving PKC to liberate PKM, which in turn phosphorylates L-type calcium channels to allow more Ca^{2+} influx. If proven, this could lend major support for this signaling loop to serve as a molecular substrate for behavioral sensitization and addiction, both of which call for positive reinforcement. This is supported by the results in Chapter 3 that carbachol-induced increases in nifedipine-sensitive Ca^{2+} currents could be blocked by PKC inhibitors. Functionally, being persistently active, PKM could fulfill all PKC-mediated processes reported to occur in addiction and motivation (Steketee, 1993;1994;1997;Steketee, et al., 1998). In addition, PKM could mediate addictive

behaviors by its role in inducing burst firing reported here as burst firing elevates terminal DA more effectively and all of these behaviors involve increased DA transmission. Furthermore, addiction is a learned behavior and PKM has been shown to facilitate learning and memory in rats (Osten, et al., 1996;Sacktor, et al., 1993). Taken together, PKM appears to be ideally positioned to affect many aspects of rewarding processing and addiction.

5.3 Future directions

As discussed in previous chapters, the L-type calcium channel-PKC (PKM) signaling pathway as a mediator of DA bursting is the major finding of this thesis work. Although our results provide fairly strong support for its role in regulating DA firing activities, there are still details left to unravel in the future.

Firstly, results in this thesis show that $Ca_v1.3$ L-type calcium channels mediate burst firing induced by L-type calcium channels, although it remains unclear whether $Ca_v1.3$ L-type calcium channels also mediate the activation and cleavage of PKCs described in Chapters 2 and 3. This can be tested using Western blotting and L-type calcium channel transgenic mice to see whether activation or inhibition of $Ca_v1.3$ L-type calcium channels affects the expression of PKM and carbachol-induced excitation.

Secondly, since the L-type calcium channel-PKC (PKM) signaling pathway is implicated in burst firing, it would be of great interest to identify the PKC substrates that lead to this firing regulation. L-type calcium channels themselves could be one candidate substrate of PKC in this signaling pathway because PKC does phosphorylate both subtypes of the L-type calcium channel at multiple serine/threonine sites. It is reasonable to examine whether L-type calcium channels themselves are the substrate of PKC (or PKM).

In addition, the M channel could be another possible candidate. The M current is a voltage-dependent, slow delayed rectifier K^+ current that is activated at subthreshold potentials and contributes to regulation of repetitive firing and excitability (Aiken, et al., 1995;Brown and Adams, 1980). M current is mediated by KCNQ-type potassium channels (Wang, et al., 1998) and immunohistochemical studies have shown that the KCNQ2 and KCNQ4 channel proteins are present in VTA neurons (Cooper, et al., 2001;Kharkovets, et al., 2000). Consistent with the presence of KCNQ channel proteins found in these anatomical studies, M current has been shown to be present in midbrain DA neurons with intracellular recording in brain slices (Lacey, et al., 1990) and clamp recording in isolated DA neurons (Koyama and Appel, 2006). Preliminary results from our lab indicate that blockade of the M current induces increased firing frequencies and even burst firing in some DA cells (unpublished data from our lab). Furthermore, M

currents have been reported to be suppressed by PKC phosphorylation (Higashida, et al., 2005). Therefore, increased PKC/PKM activity following L-type calcium channel opening could lead to phosphorylation and closing of KCNQ channels to aid burst firing.

Thirdly, the upstream events of the L-type calcium channel-PKC (PKM) signaling pathway are still unclear. Chapter 3 presents results that cholinergic activation can sequentially activate PKC and L-type calcium channels to mediate increased firing, which suggests cholinoceptors might be upstream of this L-type calcium channel-PKC signaling pathway. Whether PKC is involved in bursting induced by cholinergic activation is unclear even though this possibility clearly exists because L-type calcium channels have been shown to mediate carbachol-induced bursting (Zhang, et al., 2005).

Additionally, orexin receptors are another promising candidate. The VTA receives input from the lateral hypothalamic area, including projections from neurons containing the neurotransmitters orexin A and B (Fadel and Deutch, 2002), some of which are passing fibers and some directly synapse onto VTA neurons (Balcita-Pedicino and Sesack, 2007). Also mRNAs for orexin A and B receptors are expressed in the VTA (Korotkova, et al., 2003). In addition, the activity of VTA DA neurons has been shown to be regulated by orexin transmission. For example, activation of orexin A or B receptors in the VTA has been shown to induce DA neuron excitation, displaying increased firing

frequencies and in some cells, burst firing (Korotkova, et al., 2003). In addition, it has been reported that orexin A activates PKC to increase internal Ca^{2+} concentration in DA neurons of the VTA (Uramura, et al., 2001) and orexin B augments L-type calcium currents via a PKC signaling pathway (Xu, et al., 2003). It will be interesting to know whether an orexin-PKC-L-type calcium channel signaling pathway is involved in the regulation of DA firing activities.

References

Aiken, S.P., Lampe, B.J., Murphy, P.A. and Brown, B.S., 1995. Reduction of spike frequency adaptation and blockade of M-current in rat CA1 pyramidal neurons by linopirdine (DuP 996), a neurotransmitter release enhancer. Br J Pharmacol 115, 1163-1168.

Akaoka, H., Charlety, P., Saunier, C.F., Buda, M. and Chouvet, G., 1992. Inhibition of nigral dopamine neurons by systemic and local apomorphine: possible contribution of dendritic autoreceptors. Neuroscience 49, 879-891.

Al, Z. and Cohen, C.M., 1993. Phorbol 12-myristate 13-acetate-stimulated phosphorylation of erythrocyte membrane skeletal proteins is blocked by calpain inhibitors: possible role of protein kinase M. Biochem J 296 (Pt 3), 675-683.

Amini, B., Clark, J.W., Jr. and Canavier, C.C., 1999. Calcium dynamics underlying pacemaker-like and burst firing oscillations in midbrain dopaminergic neurons: a computational study. J Neurophysiol 82, 2249-2261.

Anden, N.E., Carlsson, A., Dahlstroem, A., Fuxe, K., Hillarp, N.A. and Larsson, K., 1964. Demonstration and Mapping out of Nigro-Neostriatal Dopamine Neurons. Life Sci 3, 523-530.

Anden, N.E., Dahlstrom, A., Fuxe, K. and Larsson, K., 1965. Mapping out of catecholamine and 5-hydroxytryptamine neurons innervating the telencephalon and diencephalon. Life Sci 4, 1275-1279.

Andersen, S.L. and Gazzara, R.A., 1996. Effects of (-)-sulpiride on dopamine release in striatum of developing rats: degree of depolarization influences responsiveness. J Neurochem 67, 1931-1937.

Andersen, S.L., Rutstein, M., Benzo, J.M., Hostetter, J.C. and Teicher, M.H., 1997. Sex differences in dopamine receptor overproduction and elimination. Neuroreport 8, 1495-1498.

Arnsten, A.F. and Li, B.M., 2005. Neurobiology of executive functions: catecholamine influences on prefrontal cortical functions. Biol Psychiatry 57, 1377-1384.

Bachtell, R.K., Whisler, K., Karanian, D. and Self, D.W., 2005. Effects of intra-nucleus accumbens shell administration of dopamine agonists and antagonists on cocaine-taking and cocaine-seeking behaviors in the rat. Psychopharmacology (Berl) 183, 41-53.

Balcita-Pedicino, J.J. and Sesack, SR, 2007. Orexin axons in the rat ventral tegmental area synapse infrequently onto dopamine and gamma-aminobutyric acid neurons. J Comp Neurol 503, 668-84.

Balfour, D.J., Wright, A.E., Benwell, M.E. and Birrell, C.E., 2000. The putative role of extra-synaptic mesolimbic dopamine in the neurobiology of nicotine dependence. Behav Brain Res 113, 73-83.

Baroudi, G., Qu, Y., Ramadan, O., Chahine, M. and Boutjdir, M., 2006. Protein kinase C activation inhibits Cav1.3 calcium channel at NH2-terminal serine 81 phosphorylation site. Am J Physiol Heart Circ Physiol 291, H1614-1622.

Bayer, V.E. and Pickel, V.M., 1991. GABA-labeled terminals form proportionally more synapses with dopaminergic neurons containing low densities of tyrosine hydroxylase-immunoreactivity in rat ventral tegmental area. Brain Res 559, 44-55.

Bean, A.J. and Roth, R.H., 1991. Extracellular dopamine and neurotensin in rat prefrontal cortex in vivo: effects of median forebrain bundle stimulation frequency, stimulation pattern, and dopamine autoreceptors. J Neurosci 11, 2694-2702.

Bean, B.P., 1989. Classes of calcium channels in vertebrate cells. Annu Rev Physiol 51, 367-384.

Beart, P.M., McDonald, D. and Gundlach, A.L., 1979. Mesolimbic dopaminergic neurones and somatodendritic mechanisms. Neurosci Lett 15, 165-170.

Beckstead, M.J., Grandy, D.K., Wickman, K. and Williams, J.T., 2004. Vesicular dopamine release elicits an inhibitory postsynaptic current in midbrain dopamine neurons. Neuron 42, 939-946.

Bengtson, C.P., Tozzi, A., Bernardi, G. and Mercuri, N.B., 2004. Transient receptor potential-like channels mediate metabotropic glutamate receptor EPSCs in rat dopamine neurones. J Physiol 555, 323-330.

Beninato, M. and Spencer, R.F., 1987. A cholinergic projection to the rat substantia nigra from the pedunculopontine tegmental nucleus. Brain Res 412, 169-174.

Berridge, M.J., Bootman, M.D. and Roderick, H.L., 2003. Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol 4, 517-529.

Bjorklund, A. and Dunnett, S.B., 2007. Dopamine neuron systems in the brain: an update. Trends Neurosci 30, 194-202.

Bolam, J.P., Francis, C.M. and Henderson, Z., 1991. Cholinergic input to dopaminergic neurons in the substantia nigra: a double immunocytochemical study. Neuroscience 41, 483-494.

Bonci, A., Grillner, P., Mercuri, N.B. and Bernardi, G., 1998. L-Type calcium channels mediate a slow excitatory synaptic transmission in rat midbrain dopaminergic neurons. J Neurosci 18, 6693-6703.

Bonci, A., Grillner, P., Siniscalchi, A., Mercuri, N.B. and Bernardi, G., 1997. Glutamate metabotropic receptor agonists depress excitatory and inhibitory transmission on rat mesencephalic principal neurons. Eur J Neurosci 9, 2359-2369.

Bradberry, C.W. and Roth, R.H., 1989. Cocaine increases extracellular dopamine in rat nucleus accumbens and ventral tegmental area as shown by in vivo microdialysis. Neurosci Lett 103, 97-102.

Braszko, J.J., Bannon, M.J., Bunney, B.S. and Roth, R.H., 1981. Intrastriatal kainic acid: acute effects on electrophysiological and biochemical measures of nigrostriatal dopaminergic activity. J Pharmacol Exp Ther 216, 289-293.

Brown, D.A. and Adams, P.R., 1980. Muscarinic suppression of a novel voltage-sensitive K+ current in a vertebrate neurone. Nature 283, 673-676.

Budde, T., Meuth, S. and Pape, H.C., 2002. Calcium-dependent inactivation of neuronal calcium channels. Nat Rev Neurosci 3, 873-883.

Bunney, B.S. and Aghajanian, G.K., 1977. D-Amphetamine-induced inhibition of central dopaminergic neurons: direct effect or mediated by a striatonigral feedback pathway? Adv Biochem Psychopharmacol 16, 577-582.

Bunney, B.S., Walters, J.R., Roth, R.H. and Aghajanian, G.K., 1973. Dopaminergic neurons: effect of antipsychotic drugs and amphetamine on single cell activity. J Pharmacol Exp Ther 185, 560-571.

Calabresi, P., Lacey, M.G. and North, R.A., 1989. Nicotinic excitation of rat ventral tegmental neurones in vitro studied by intracellular recording. Br J Pharmacol 98, 135-140.

Callaghan, B., Koh, S.D. and Keef, K.D., 2004. Muscarinic M2 receptor stimulation of Cav1.2b requires phosphatidylinositol 3-kinase, protein kinase C, and c-Src. Circ Res 94, 626-633.

Cardozo, D.L. and Bean, B.P., 1995. Voltage-dependent calcium channels in rat midbrain dopamine neurons: modulation by dopamine and GABAB receptors. J Neurophysiol 74, 1137-1148.

Carlin, K.P., Jones, K.E., Jiang, Z., Jordan, L.M. and Brownstone, R.M., 2000. Dendritic L-type calcium currents in mouse spinal motoneurons: implications for bistability. Eur J Neurosci 12, 1635-1646.

Carlsson, A., 1988. The current status of the dopamine hypothesis of schizophrenia. Neuropsychopharmacology 1, 179-186.

Carlsson, A., Lindqvist, M. and Magnusson, T., 1957. 3,4-Dihydroxyphenylalanine and 5-hydroxytryptophan as reserpine antagonists. Nature 180, 1200.

Carlsson, A., Lindqvist, M., Magnusson, T. and Waldeck, B., 1958. On the presence of 3-hydroxytyramine in brain. Science 127, 471.

Carr, D.B. and Sesack, S.R., 1999. Terminals from the rat prefrontal cortex synapse on mesoaccumbens VTA neurons. Ann N Y Acad Sci 877, 676-678.

Carr, D.B. and Sesack, S.R., 2000a. GABA-containing neurons in the rat ventral tegmental area project to the prefrontal cortex. Synapse 38, 114-123.

Carr, D.B. and Sesack, S.R., 2000b. Projections from the rat prefrontal cortex to the ventral tegmental area: target specificity in the synaptic associations with mesoaccumbens and mesocortical neurons. J Neurosci 20, 3864-3873.

Caulfield, M.P., 1993. Muscarinic receptors--characterization, coupling and function. Pharmacol Ther 58, 319-379.

Chahine, M., Qu, Y., Mancarella, S. and Boutjdir, M., 2008. Protein kinase C activation inhibits alpha1D L-type Ca channel: a single-channel analysis. Pflugers Arch 455, 913-919.

Chan, C.S., Guzman, J.N., Ilijic, E., Mercer, J.N., Rick, C., Tkatch, T., Meredith, G.E. and Surmeier, D.J., 2007. 'Rejuvenation' protects neurons in mouse models of Parkinson's disease. Nature 447, 1081-1086.

Chen, B.T. and Rice, M.E., 2001. Novel Ca2+ dependence and time course of somatodendritic dopamine release: substantia nigra versus striatum. J Neurosci 21, 7841-7847.

Chen, Y., Phillips, K., Minton, G. and Sher, E., 2005. GABA(B) receptor modulators potentiate baclofen-induced depression of dopamine neuron activity in the rat ventral tegmental area. Br J Pharmacol 144, 926-932.

Cheramy, A., Leviel, V. and Glowinski, J., 1981. Dendritic release of dopamine in the substantia nigra. Nature 289, 537-542.

Chergui, K., Charlety, P.J., Akaoka, H., Saunier, C.F., Brunet, J.L., Buda, M., Svensson, T.H. and Chouvet, G., 1993. Tonic activation of NMDA receptors causes spontaneous burst discharge of rat midbrain dopamine neurons in vivo. Eur J Neurosci 5, 137-144.

Chergui, K., Suaud-Chagny, M.F. and Gonon, F., 1994. Nonlinear relationship between impulse flow, dopamine release and dopamine elimination in the rat brain in vivo. Neuroscience 62, 641-645.

Christoffersen, C.L. and Meltzer, L.T., 1995. Evidence for N-methyl-D-aspartate and AMPA subtypes of the glutamate receptor on substantia nigra dopamine neurons: possible preferential role for N-methyl-D-aspartate receptors. Neuroscience 67, 373-381.

Chu, Y., Kompoliti, K., Cochran, E.J., Mufson, E.J. and Kordower, J.H., 2002. Age-related decreases in Nurr1 immunoreactivity in the human substantia nigra. J Comp Neurol 450, 203-214.

Chuhma, N., Zhang, H., Masson, J., Zhuang, X., Sulzer, D., Hen, R., Rayport, S., 2004. Dopamine neurons mediate a fast excitatory signal via their glutamatergic synapses. J Neurosci 24, 972-81.

Clark, D. and Chiodo, L.A., 1988. Electrophysiological and pharmacological characterization of identified nigrostriatal and mesoaccumbens dopamine neurons in the rat. Synapse 2, 474-485.

Clarke, P.B., Hommer, D.W., Pert, A. and Skirboll, L.R., 1987. Innervation of substantia nigra neurons by cholinergic afferents from pedunculopontine nucleus in the rat: neuroanatomical and electrophysiological evidence. Neuroscience 23, 1011-1019.

Clarke, P.B. and Pert, A., 1985. Autoradiographic evidence for nicotine receptors on nigrostriatal and mesolimbic dopaminergic neurons. Brain Res 348, 355-358.

Clarke, P.B., Schwartz, R.D., Paul, S.M., Pert, C.B. and Pert, A., 1985. Nicotinic binding in rat brain: autoradiographic comparison of [3H]acetylcholine, [3H]nicotine, and [125I]-alpha-bungarotoxin. J Neurosci 5, 1307-1315.

Cooper, D.C., 2002. The significance of action potential bursting in the brain reward circuit. Neurochem Int 41, 333-340.

Cooper, E.C., Harrington, E., Jan, Y.N. and Jan, L.Y., 2001. M channel KCNQ2 subunits are localized to key sites for control of neuronal network oscillations and synchronization in mouse brain. J Neurosci 21, 9529-9540.

Cornwall, J., Cooper, J.D. and Phillipson, O.T., 1990. Afferent and efferent connections of the laterodorsal tegmental nucleus in the rat. Brain Res Bull 25, 271-284.

Corrigall, W.A., Coen, K.M. and Adamson, K.L., 1994. Self-administered nicotine activates the mesolimbic dopamine system through the ventral tegmental area. Brain Res 653, 278-284.

Corrigall, W.A., Franklin, K.B., Coen, K.M. and Clarke, P.B., 1992. The mesolimbic dopaminergic system is implicated in the reinforcing effects of nicotine. Psychopharmacology (Berl) 107, 285-289.

Cressman, C.M., Mohan, P.S., Nixon, R.A. and Shea, T.B., 1995. Proteolysis of protein kinase C: mM and microM calcium-requiring calpains have different abilities to generate, and degrade the free catalytic subunit, protein kinase M. FEBS Lett 367, 223-227.

Cruz, H.G., Ivanova, T., Lunn, M.L., Stoffel, M., Slesinger, P.A. and Luscher, C., 2004. Bi-directional effects of GABA(B) receptor agonists on the mesolimbic dopamine system. Nat Neurosci 7, 153-159.

Cubeddu, L.X. and Hoffmann, I.S., 1982. Operational characteristics of the inhibitory feedback mechanism for regulation of dopamine release via presynaptic receptors. J Pharmacol Exp Ther 223, 497-501.

Dani, J.A., Ji, D. and Zhou, F.M., 2001. Synaptic plasticity and nicotine addiction. Neuron 31, 349-352.

Daniels, G.M. and Amara, S.G., 1999. Regulated trafficking of the human dopamine transporter. Clathrin-mediated internalization and lysosomal degradation in response to phorbol esters. J Biol Chem 274, 35794-35801.

Dash, P.K., Moore, A.N., Kobori, N. and Runyan, J.D., 2007. Molecular activity underlying working memory. Learn Mem 14, 554-563.

Davila, V., Yan, Z., Craciun, L.C., Logothetis, D. and Sulzer, D., 2003. D3 dopamine autoreceptors do not activate G-protein-gated inwardly rectifying potassium channel currents in substantia nigra dopamine neurons. J Neurosci 23, 5693-5697.

Descarries, L., Berube-Carriere, N., Riad, M., Bo, G.D., Mendez, J.A., Trudeau, L.E., 2008. Glutamate in dopamine neurons: synaptic versus diffuse transmission. Brain Res Rev 58, 290-302.

Di Chiara, G., 1995. The role of dopamine in drug abuse viewed from the perspective of its role in motivation. Drug Alcohol Depend 38, 95-137.

Di Chiara, G. and Imperato, A., 1988. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc Natl Acad Sci U S A 85, 5274-5278.

Di Loreto, S., Florio, T. and Scarnati, E., 1992. Evidence that non-NMDA receptors are involved in the excitatory pathway from the pedunculopontine region to nigrostriatal dopaminergic neurons. Exp Brain Res 89, 79-86.

Druhan, J.P., Fibiger, H.C. and Phillips, A.G., 1989. Differential effects of cholinergic drugs on discriminative cues and self-stimulation produced by electrical stimulation of the ventral tegmental area. Psychopharmacology (Berl) 97, 331-338.

Durante, P., Cardenas, C.G., Whittaker, J.A., Kitai, S.T. and Scroggs, R.S., 2004. Low-threshold L-type calcium channels in rat dopamine neurons. J Neurophysiol 91, 1450-1454.

Dzhura, I., Wu, Y., Colbran, R.J., Balser, J.R. and Anderson, M.E., 2000. Calmodulin kinase determines calcium-dependent facilitation of L-type calcium channels. Nat Cell Biol 2, 173-177.

Einhorn, L.C., Johansen, P.A. and White, F.J., 1988. Electrophysiological effects of cocaine in the mesoaccumbens dopamine system: studies in the ventral tegmental area. J Neurosci 8, 100-112.

Emborg, M.E., Ma, S.Y., Mufson, E.J., Levey, A.I., Taylor, M.D., Brown, W.D., Holden, J.E. and Kordower, J.H., 1998. Age-related declines in nigral neuronal

function correlate with motor impairments in rhesus monkeys. J Comp Neurol 401, 253-265.

Engberg, G. and Nissbrandt, H., 1993. gamma-Hydroxybutyric acid (GHBA) induces pacemaker activity and inhibition of substantia nigra dopamine neurons by activating GABAB-receptors. Naunyn Schmiedebergs Arch Pharmacol 348, 491-497.

Erhardt, S., Andersson, B., Nissbrandt, H. and Engberg, G., 1998. Inhibition of firing rate and changes in the firing pattern of nigral dopamine neurons by gamma-hydroxybutyric acid (GHBA) are specifically induced by activation of GABA(B) receptors. Naunyn Schmiedebergs Arch Pharmacol 357, 611-619.

Erhardt, S. and Engberg, G., 2000. Excitation of nigral dopamine neurons by the GABA(A) receptor agonist muscimol is mediated via release of glutamate. Life Sci 67, 1901-1911.

Erhardt, S., Mathe, J.M., Chergui, K., Engberg, G. and Svensson, T.H., 2002. GABA(B) receptor-mediated modulation of the firing pattern of ventral tegmental area dopamine neurons in vivo. Naunyn Schmiedebergs Arch Pharmacol 365, 173-180.

Ertel, E.A., Campbell, K.P., Harpold, M.M., Hofmann, F., Mori, Y., Perez-Reyes, E., Schwartz, A., Snutch, T.P., Tanabe, T., Birnbaumer, L., Tsien, R.W. and Catterall, W.A., 2000. Nomenclature of voltage-gated calcium channels. Neuron 25, 533-535.

Fadel, J. and Deutch, A.Y., 2002. Anatomical substrates of orexin-dopamine interactions: lateral hypothalamic projections to the ventral tegmental area. Neuroscience 111, 379-387.

Fayyazuddin A., Villarroel A., Le Goff A., Lerma J. and Neyton J., 2000. Four residues of the extracellular N-terminal domain of the NR2A subunit control high-affinity Zn2+ binding to NMDA receptors. Neuron 25, 683-94.

Fiorillo, C.D. and Williams, J.T., 1998. Glutamate mediates an inhibitory postsynaptic potential in dopamine neurons. Nature 394, 78-82.

Floresco, S.B. and Phillips, A.G., 2001. Delay-dependent modulation of memory retrieval by infusion of a dopamine D1 agonist into the rat medial prefrontal cortex. Behav Neurosci 115, 934-939.

Floresco, S.B., Todd, C.L. and Grace, A.A., 2001. Glutamatergic afferents from the hippocampus to the nucleus accumbens regulate activity of ventral tegmental area dopamine neurons. J Neurosci 21, 4915-4922.

Floresco, S.B., West, A.R., Ash, B., Moore, H. and Grace, A.A., 2003. Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. Nat Neurosci 6, 968-973.

Forster, G.L. and Blaha, C.D., 2000. Laterodorsal tegmental stimulation elicits dopamine efflux in the rat nucleus accumbens by activation of acetylcholine and glutamate receptors in the ventral tegmental area. Eur J Neurosci 12, 3596-3604.

Forster, G.L., Yeomans, J.S., Takeuchi, J. and Blaha, C.D., 2002. M5 muscarinic receptors are required for prolonged accumbal dopamine release after electrical stimulation of the pons in mice. J Neurosci 22, RC190.

Freeman, A.S. and Bunney, B.S., 1987. Activity of A9 and A10 dopaminergic neurons in unrestrained rats: further characterization and effects of apomorphine and cholecystokinin. Brain Res 405, 46-55.

Freeman, A.S., Kelland, M.D., Rouillard, C. and Chiodo, L.A., 1989. Electrophysiological characteristics and pharmacological responsiveness of midbrain dopaminergic neurons of the aged rat. J Pharmacol Exp Ther 249, 790-797.

Frere, S.G., Kuisle, M. and Luthi, A., 2004. Regulation of recombinant and native hyperpolarization-activated cation channels. Mol Neurobiol 30, 279-305.

Fu, L.Y., Wang, F., Chen, X.S., Zhou, H.Y., Yao, W.X., Xia, G.J. and Jiang, M.X., 2003. Perforated patch recording of L-type calcium current with beta-escin in guinea pig ventricular myocytes. Acta Pharmacol Sin 24, 1094-1098.

Gahring, L.C., Persiyanov, K. and Rogers, S.W., 2004. Neuronal and astrocyte expression of nicotinic receptor subunit beta4 in the adult mouse brain. J Comp Neurol 468, 322-333.

Gariano, R.F. and Groves, P.M., 1988. Burst firing induced in midbrain dopamine neurons by stimulation of the medial prefrontal and anterior cingulate cortices. Brain Res 462, 194-198.

Gariano, R.F., Tepper, J.M., Sawyer, S.F., Young, S.J. and Groves, P.M., 1989. Mesocortical dopaminergic neurons. I. Electrophysiological properties and evidence for soma-dendritic autoreceptors. Brain Res Bull 22, 511-516.

Garris, P.A., Ciolkowski, E.L., Pastore, P. and Wightman, R.M., 1994. Efflux of dopamine from the synaptic cleft in the nucleus accumbens of the rat brain. J Neurosci 14, 6084-6093.

Gavazzo, P., Mazzolini, M., Tedesco, M. and Marchetti, C., 2006. Nickel differentially affects NMDA receptor channels in developing cultured rat neurons. Brain Res 1078, 71-9.

Georges, F. and Aston-Jones, G., 2001. Potent regulation of midbrain dopamine neurons by the bed nucleus of the stria terminalis. J Neurosci 21, RC160.

Gereau, R.W.t. and Conn, P.J., 1995. Multiple presynaptic metabotropic glutamate receptors modulate excitatory and inhibitory synaptic transmission in hippocampal area CA1. J Neurosci 15, 6879-6889.

Gerfen, C.R., Herkenham, M. and Thibault, J., 1987. The neostriatal mosaic: II. Patch- and matrix-directed mesostriatal dopaminergic and non-dopaminergic systems. J Neurosci 7, 3915-3934.

German, D.C. and Manaye, K.F., 1993. Midbrain dopaminergic neurons (nuclei A8, A9, and A10): three-dimensional reconstruction in the rat. J Comp Neurol 331, 297-309.

Giordano, T.P., 3rd, Satpute, S.S., Striessnig, J., Kosofsky, B.E. and Rajadhyaksha, A.M., 2006. Up-regulation of dopamine D(2)L mRNA levels in the ventral tegmental area and dorsal striatum of amphetamine-sensitized C57BL/6 mice: role of Ca(v)1.3 L-type Ca(2+) channels. J Neurochem 99, 1197-1206.

Goll, D.E., Thompson, V.F., Li, H., Wei, W. and Cong, J., 2003. The calpain system. Physiol Rev 83, 731-801.

Gomez, L.L., Alam, S., Smith, K.E., Horne, E. and Dell'Acqua, M.L., 2002. Regulation of A-kinase anchoring protein 79/150-cAMP-dependent protein kinase postsynaptic targeting by NMDA receptor activation of calcineurin and remodeling of dendritic actin. J Neurosci 22, 7027-7044.

Gonon, F.G., 1988. Nonlinear relationship between impulse flow and dopamine released by rat midbrain dopaminergic neurons as studied by in vivo electrochemistry. Neuroscience 24, 19-28.

Gonon, F.G. and Buda, M.J., 1985. Regulation of dopamine release by impulse flow and by autoreceptors as studied by in vivo voltammetry in the rat striatum. Neuroscience 14, 765-774.

Goto, Y., Otani, S. and Grace, A.A., 2007. The Yin and Yang of dopamine release: a new perspective. Neuropharmacology 53, 583-587.

Grace, A.A., 2000. The tonic/phasic model of dopamine system regulation and its implications for understanding alcohol and psychostimulant craving. Addiction 95 Suppl 2, S119-128.

Grace, A.A. and Bunney, B.S., 1979. Paradoxical GABA excitation of nigral dopaminergic cells: indirect mediation through reticulata inhibitory neurons. Eur J Pharmacol 59, 211-218.

Grace, A.A. and Bunney, B.S., 1984a. The control of firing pattern in nigral dopamine neurons: burst firing. J Neurosci 4, 2877-2890.

Grace, A.A. and Bunney, B.S., 1984b. The control of firing pattern in nigral dopamine neurons: single spike firing. J Neurosci 4, 2866-2876.

Grace, A.A. and Bunney, B.S., 1985. Opposing effects of striatonigral feedback pathways on midbrain dopamine cell activity. Brain Res 333, 271-284.

Grace, A.A. and Onn, S.P., 1989. Morphology and electrophysiological properties of immunocytochemically identified rat dopamine neurons recorded in vitro. J Neurosci 9, 3463-3481.

Grenhoff, J., Aston-Jones, G. and Svensson, T.H., 1986. Nicotinic effects on the firing pattern of midbrain dopamine neurons. Acta Physiol Scand 128, 351-358.

Grenhoff, J., Ugedo, L. and Svensson, T.H., 1988. Firing patterns of midbrain dopamine neurons: differences between A9 and A10 cells. Acta Physiol Scand 134, 127-132.

Grillner, P., Bonci, A., Svensson, T.H., Bernardi, G. and Mercuri, N.B., 1999. Presynaptic muscarinic (M3) receptors reduce excitatory transmission in dopamine neurons of the rat mesencephalon. Neuroscience 91, 557-565.

Grillner, P. and Mercuri, N.B., 2002. Intrinsic membrane properties and synaptic inputs regulating the firing activity of the dopamine neurons. Behav Brain Res 130, 149-169.

Groenewegen, H.J., Berendse, H.W. and Haber, S.N., 1993. Organization of the output of the ventral striatopallidal system in the rat: ventral pallidal efferents. Neuroscience 57, 113-142.

Grosshans, D.R., Clayton, D.A., Coultrap, S.J. and Browning, M.D., 2002. LTP leads to rapid surface expression of NMDA but not AMPA receptors in adult rat CA1. Nat Neurosci 5, 27-33.

Haller, V.L., Bernstein, M.A. and Welch, S.P., 2008. Chronic morphine treatment decreases the Cav1.3 subunit of the L-type calcium channel. Eur J Pharmacol 578, 101-107.

Hell, J.W., Westenbroek, R.E., Warner, C., Ahlijanian, M.K., Prystay, W., Gilbert, M.M., Snutch, T.P. and Catterall, W.A., 1993. Identification and differential subcellular localization of the neuronal class C and class D L-type calcium channel alpha 1 subunits. J Cell Biol 123, 949-962.

Henderson, Z. and Sherriff, F.E., 1991. Distribution of choline acetyltransferase immunoreactive axons and terminals in the rat and ferret brainstem. J Comp Neurol 314, 147-163.

Herrero, I., Miras-Portugal, M.T. and Sanchez-Prieto, J., 1992. Positive feedback of glutamate exocytosis by metabotropic presynaptic receptor stimulation. Nature 360, 163-166.

Higashida, H., Hoshi, N., Zhang, J.S., Yokoyama, S., Hashii, M., Jin, D., Noda, M. and Robbins, J., 2005. Protein kinase C bound with A-kinase anchoring protein is involved in muscarinic receptor-activated modulation of M-type KCNQ potassium channels. Neurosci Res 51, 231-234.

Holton, K.L., Loder, M.K. and Melikian, H.E., 2005. Nonclassical, distinct endocytic signals dictate constitutive and PKC-regulated neurotransmitter transporter internalization. Nat Neurosci 8, 881-888.

Hoogland, T.M. and Saggau, P., 2004. Facilitation of L-type Ca2+ channels in dendritic spines by activation of beta2 adrenergic receptors. J Neurosci 24, 8416-8427.

Horn, A.S., 1990. Dopamine uptake: a review of progress in the last decade. Prog Neurobiol 34, 387-400.

Howes, O.D. and Kapur, S., 2009. The dopamine hypothesis of schizophrenia: Version III - the final common pathway. Schizophr Bull 35, 549-562.

Hsiao, C.F., Del Negro, C.A., Trueblood, P.R. and Chandler, S.H., 1998. Ionic basis for serotonin-induced bistable membrane properties in guinea pig trigeminal motoneurons. J Neurophysiol 79, 2847-2856.

Hudmon, A., Schulman, H., Kim, J., Maltez, J.M., Tsien, R.W. and Pitt, G.S., 2005. CaMKII tethers to L-type Ca2+ channels, establishing a local and dedicated integrator of Ca2+ signals for facilitation. J Cell Biol 171, 537-547.

Hug, H. and Sarre, T.F., 1993. Protein kinase C isoenzymes: divergence in signal transduction? Biochem J 291 (Pt 2), 329-343.

Hulme, E.C., Birdsall, N.J. and Buckley, N.J., 1990. Muscarinic receptor subtypes. Annu Rev Pharmacol Toxicol 30, 633-673.

Huneke, R., Zitzelsberger, D., Fassl, J., Jungling, E., Brose, S., Buhre, W., Rossaint, R. and Luckhoff, A., 2004. Temperature-independent Inhibition of L-type calcium currents by halothane and sevoflurane in human atrial cardiomyocytes. Anesthesiology 101, 409-416.

Hyland, B.I., Reynolds, J.N., Hay, J., Perk, C.G. and Miller, R., 2002. Firing modes of midbrain dopamine cells in the freely moving rat. Neuroscience 114, 475-492.

Ikemoto, S., 2007. Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. Brain Res Rev 56, 27-78.

Innis, R.B. and Aghajanian, G.K., 1987. Pertussis toxin blocks autoreceptor-mediated inhibition of dopaminergic neurons in rat substantia nigra. Brain Res 411, 139-143.

Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y., 1977. Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain. J Biol Chem 252, 7610-7616.

Johnson, S.W. and North, R.A., 1992a. Opioids excite dopamine neurons by hyperpolarization of local interneurons. J Neurosci 12, 483-488.

Johnson, S.W. and North, R.A., 1992b. Two types of neurone in the rat ventral tegmental area and their synaptic inputs. J Physiol 450, 455-468.

Johnson, S.W. and Seutin, V., 1997. Bicuculline methiodide potentiates NMDA-dependent burst firing in rat dopamine neurons by blocking apamin-sensitive Ca2+-activated K+ currents. Neurosci Lett 231, 13-16.

Johnson, S.W., Seutin, V. and North, R.A., 1992. Burst firing in dopamine neurons induced by N-methyl-D-aspartate: role of electrogenic sodium pump. Science 258, 665-667.

Johnson, S.W. and Wu, Y.N., 2004. Multiple mechanisms underlie burst firing in rat midbrain dopamine neurons in vitro. Brain Res 1019, 293-296.

Jomphe, C., Bourque, M.J., Fortin, G.D., St-Gelais, F., Okano, H., Kobayashi, K. and Trudeau, L.E., 2005. Use of TH-EGFP transgenic mice as a source of identified dopaminergic neurons for physiological studies in postnatal cell culture. J Neurosci Methods 146, 1-12.

Kalivas, P.W. and Duffy, P., 1991. A comparison of axonal and somatodendritic dopamine release using in vivo dialysis. J Neurochem 56, 961-967.

Kamp, T.J. and Hell, J.W., 2000. Regulation of cardiac L-type calcium channels by protein kinase A and protein kinase C. Circ Res 87, 1095-1102.

Kauer, J.A. and Malenka, R.C., 2007. Synaptic plasticity and addiction. Nat Rev Neurosci 8, 844-858.

Kelley, A.E., 2004. Memory and addiction: shared neural circuitry and molecular mechanisms. Neuron 44, 161-179.

Kharkovets, T., Hardelin, J.P., Safieddine, S., Schweizer, M., El-Amraoui, A., Petit, C. and Jentsch, T.J., 2000. KCNQ4, a K+ channel mutated in a form of dominant deafness, is expressed in the inner ear and the central auditory pathway. Proc Natl Acad Sci U S A 97, 4333-4338.

Kilpatrick, M.R., Rooney, M.B., Michael, D.J. and Wightman, R.M., 2000. Extracellular dopamine dynamics in rat caudate-putamen during experimenter-delivered and intracranial self-stimulation. Neuroscience 96, 697-706.

Kim, S.H., Choi, Y.M., Chung, S., Uhm, D.Y. and Park, M.K., 2004. Two different Ca2+-dependent inhibitory mechanisms of spontaneous firing by glutamate in dopamine neurons. J Neurochem 91, 983-995.

Kishimoto, A., Kajikawa, N., Shiota, M. and Nishizuka, Y., 1983. Proteolytic activation of calcium-activated, phospholipid-dependent protein kinase by calcium-dependent neutral protease. J Biol Chem 258, 1156-1164.

Kitai, S.T., Shepard, P.D., Callaway, J.C. and Scroggs, R., 1999. Afferent modulation of dopamine neuron firing patterns. Curr Opin Neurobiol 9, 690-697.

Klein, R.C. and Yakel, J.L., 2006. Functional somato-dendritic alpha7-containing nicotinic acetylcholine receptors in the rat basolateral amygdala complex. J Physiol 576, 865-872.

Kohler, M., Hirschberg, B., Bond, C.T., Kinzie, J.M., Marrion, N.V., Maylie, J. and Adelman, J.P., 1996. Small-conductance, calcium-activated potassium channels from mammalian brain. Science 273, 1709-1714.

Koob, G.F., 2000. Neurobiology of addiction. Toward the development of new therapies. Ann N Y Acad Sci 909, 170-185.

Koob, G.F., Sanna, P.P. and Bloom, F.E., 1998. Neuroscience of addiction. Neuron 21, 467-476.

Korotkova, T.M., Sergeeva, O.A., Eriksson, K.S., Haas, H.L. and Brown, R.E., 2003. Excitation of ventral tegmental area dopaminergic and nondopaminergic neurons by orexins/hypocretins. J Neurosci 23, 7-11.

Kosaka, T., Kosaka, K., Hataguchi, Y., Nagatsu, I., Wu, J.Y., Ottersen, O.P., Storm-Mathisen, J. and Hama, K., 1987. Catecholaminergic neurons containing GABA-like and/or glutamic acid decarboxylase-like immunoreactivities in various brain regions of the rat. Exp Brain Res 66, 191-210.

Koyama, S. and Appel, S.B., 2006. Characterization of M-current in ventral tegmental area dopamine neurons. J Neurophysiol 96, 535-543.

Lacey, M.G., Calabresi, P. and North, R.A., 1990. Muscarine depolarizes rat substantia nigra zona compacta and ventral tegmental neurons in vitro through M1-like receptors. J Pharmacol Exp Ther 253, 395-400.

Lacey, M.G., Mercuri, N.B. and North, R.A., 1987. Dopamine acts on D2 receptors to increase potassium conductance in neurones of the rat substantia nigra zona compacta. J Physiol 392, 397-416.

Lacey, M.G., Mercuri, N.B. and North, R.A., 1989. Two cell types in rat substantia nigra zona compacta distinguished by membrane properties and the actions of dopamine and opioids. J Neurosci 9, 1233-1241.

Lammel, S., Hetzel, A., Hackel, O., Jones, I., Liss, B., Roeper, J., 2008. Unique properties of mesoprefrontal neurons within a dual mesocorticolimbic dopamine system. Neuron 57, 760-773.

Lavin, A., Nogueira, L., Lapish, C.C., Wightman, R.M., Phillips, P.E., Seamans, J.K., 2005. Mesocortical dopamine neurons operate in distinct temporal domains using multimodal signaling. J Neurosci 25, 5013-5023.

Lavin, M.A. and Drucker-Colin, R., 1991. Ontogeny of the electrophysiological activity of dopaminergic cells with special reference to the influence of adrenal medullary grafts on aging. Brain Res 545, 164-170.

Laviola, G., Pascucci, T. and Pieretti, S., 2001. Striatal dopamine sensitization to D-amphetamine in periadolescent but not in adult rats. Pharmacol Biochem Behav 68, 115-124.

Laviolette, S.R., 2007. Dopamine modulation of emotional processing in cortical and subcortical neural circuits: evidence for a final common pathway in schizophrenia? Schizophr Bull 33, 971-981.

Lavoie, B. and Parent, A., 1994a. Pedunculopontine nucleus in the squirrel monkey: cholinergic and glutamatergic projections to the substantia nigra. J Comp Neurol 344, 232-241.

Lavoie, B. and Parent, A., 1994b. Pedunculopontine nucleus in the squirrel monkey: distribution of cholinergic and monoaminergic neurons in the mesopontine tegmentum with evidence for the presence of glutamate in cholinergic neurons. J Comp Neurol 344, 190-209.

Lavoie, B. and Parent, A., 1994c. Pedunculopontine nucleus in the squirrel monkey: projections to the basal ganglia as revealed by anterograde tract-tracing methods. J Comp Neurol 344, 210-231.

Le Moal, M. and Simon, H., 1991. Mesocorticolimbic dopaminergic network: functional and regulatory roles. Physiol Rev 71, 155-234.

Lee, T.S., Karl, R., Moosmang, S., Lenhardt, P., Klugbauer, N., Hofmann, F., Kleppisch, T. and Welling, A., 2006. Calmodulin kinase II is involved in voltage-dependent facilitation of the L-type Cav1.2 calcium channel: Identification of the phosphorylation sites. J Biol Chem 281, 25560-25567.

Lena, C., de Kerchove D'Exaerde, A., Cordero-Erausquin, M., Le Novere, N., del Mar Arroyo-Jimenez, M. and Changeux, J.P., 1999. Diversity and distribution of

nicotinic acetylcholine receptors in the locus ceruleus neurons. Proc Natl Acad Sci U S A 96, 12126-12131.

Levitan, I.B., 2006. Signaling protein complexes associated with neuronal ion channels. Nat Neurosci 9, 305-310.

Lewis, D.A., Sesack, S.R., Levey, A.I. and Rosenberg, D.R., 1998. Dopamine axons in primate prefrontal cortex: specificity of distribution, synaptic targets, and development. Adv Pharmacol 42, 703-706.

Licata, S.C., Freeman, A.Y., Pierce-Bancroft, A.F. and Pierce, R.C., 2000. Repeated stimulation of L-type calcium channels in the rat ventral tegmental area mimics the initiation of behavioral sensitization to cocaine. Psychopharmacology (Berl) 152, 110-118.

Licata, S.C., Schmidt, H.D. and Pierce, R.C., 2004. Suppressing calcium/calmodulin-dependent protein kinase II activity in the ventral tegmental area enhances the acute behavioural response to cocaine but attenuates the initiation of cocaine-induced behavioural sensitization in rats. Eur J Neurosci 19, 405-414.

Liljelund, P., Netzeband, J.G. and Gruol, D.L., 2000. L-Type calcium channels mediate calcium oscillations in early postnatal Purkinje neurons. J Neurosci 20, 7394-7403.

Lipscombe, D., Helton, T.D. and Xu, W., 2004. L-type calcium channels: the low down. J Neurophysiol 92, 2633-2641.

Lisman, J.E. and Grace, A.A., 2005. The hippocampal-VTA loop: controlling the entry of information into long-term memory. Neuron 46, 703-713.

Liss, B., Franz, O., Sewing, S., Bruns, R., Neuhoff, H. and Roeper, J., 2001. Tuning pacemaker frequency of individual dopaminergic neurons by Kv4.3L and KChip3.1 transcription. Embo J 20, 5715-5724.

Liu, W.S. and Heckman, C.A., 1998. The sevenfold way of PKC regulation. Cell Signal 10, 529-542.

Liu, Y., Dore, J. and Chen, X., 2007. Calcium influx through L-type channels generates protein kinase M to induce burst firing of dopamine cells in the rat ventral tegmental area. J Biol Chem 282, 8594-8603.

Loder, M.K. and Melikian, H.E., 2003. The dopamine transporter constitutively internalizes and recycles in a protein kinase C-regulated manner in stably transfected PC12 cell lines. J Biol Chem 278, 22168-22174.

Lodge, D.J. and Grace, A.A., 2006. The laterodorsal tegmentum is essential for burst firing of ventral tegmental area dopamine neurons. Proc Natl Acad Sci U S A 103, 5167-5172.

Lokwan, S.J., Overton, P.G., Berry, M.S. and Clark, D., 1999. Stimulation of the pedunculopontine tegmental nucleus in the rat produces burst firing in A9 dopaminergic neurons. Neuroscience 92, 245-254.

Louis, M. and Clarke, P.B., 1998. Effect of ventral tegmental 6-hydroxydopamine lesions on the locomotor stimulant action of nicotine in rats. Neuropharmacology 37, 1503-1513.

Lynd-Balta, E. and Haber, S.N., 1994. The organization of midbrain projections to the striatum in the primate: sensorimotor-related striatum versus ventral striatum. Neuroscience 59, 625-640.

Majewski, H. and Iannazzo, L., 1998. Protein kinase C: a physiological mediator of enhanced transmitter output. Prog Neurobiol 55, 463-475.

Manley, L.D., Kuczenski, R., Segal, D.S., Young, S.J. and Groves, P.M., 1992. Effects of frequency and pattern of medial forebrain bundle stimulation on caudate dialysate dopamine and serotonin. J Neurochem 58, 1491-1498.

Mansvelder, H.D. and McGehee, D.S., 2000. Long-term potentiation of excitatory inputs to brain reward areas by nicotine. Neuron 27, 349-357.

Margolis, E.B., Lock, H., Hjelmstad, G.O. and Fields, H.L., 2006. The ventral tegmental area revisited: is there an electrophysiological marker for dopaminergic neurons? J Physiol 577, 907-924.

Marinelli, M. and White, F.J., 2000. Enhanced vulnerability to cocaine self-administration is associated with elevated impulse activity of midbrain dopamine neurons. J Neurosci 20, 8876-8885.

Mathon, D.S., Kamal, A., Smidt, M.P. and Ramakers, G.M., 2003. Modulation of cellular activity and synaptic transmission in the ventral tegmental area. Eur J Pharmacol 480, 97-115.

McArdle, C.A. and Conn, P.M., 1989. Use of protein kinase C-depleted cells for investigation of the role of protein kinase C in stimulus-response coupling in the pituitary. Methods Enzymol 168, 287-301.

McKinney, B.C. and Murphy, G.G., 2006. The L-Type voltage-gated calcium channel Cav1.3 mediates consolidation, but not extinction, of contextually conditioned fear in mice. Learn Mem 13, 584-589.

Melikian, H.E. and Buckley, K.M., 1999. Membrane trafficking regulates the activity of the human dopamine transporter. J Neurosci 19, 7699-7710.

Meltzer, L.T., Serpa, K.A. and Christoffersen, C.L., 1997. Metabotropic glutamate receptor-mediated inhibition and excitation of substantia nigra dopamine neurons. Synapse 26, 184-193.

Mena-Segovia, J., Winn, P. and Bolam, J.P., 2008. Cholinergic modulation of midbrain dopaminergic systems. Brain Res Rev.

Mendez, J.A., Bourque, M.J., Dal Bo, G., Bourdeau, M.L., Danik, M., Williams, S., Lacaille, J.C., Trudeau, L.E., 2008. Developmental and target-dependent regulation of vesicular glutamate transporter expression by dopamine neurons. J Neurosci 28, 6309-6318.

Mercuri, N.B., Bonci, A., Calabresi, P., Stefani, A. and Bernardi, G., 1995. Properties of the hyperpolarization-activated cation current Ih in rat midbrain dopaminergic neurons. Eur J Neurosci 7, 462-469.

Mercuri, N.B., Bonci, A., Calabresi, P., Stratta, F., Stefani, A. and Bernardi, G., 1994. Effects of dihydropyridine calcium antagonists on rat midbrain dopaminergic neurones. Br J Pharmacol 113, 831-838.

Mercuri, N.B., Calabresi, P. and Bernardi, G., 1992a. The electrophysiological actions of dopamine and dopaminergic drugs on neurons of the substantia nigra pars compacta and ventral tegmental area. Life Sci 51, 711-718.

Mercuri, N.B., Grillner, P. and Bernardi, G., 1996. N-methyl-D-aspartate receptors mediate a slow excitatory postsynaptic potential in the rat midbrain dopaminergic neurons. Neuroscience 74, 785-792.

Mercuri, N.B., Stratta, F., Calabresi, P. and Bernardi, G., 1992b. Electrophysiological evidence for the presence of ionotropic and metabotropic excitatory amino acid receptors on dopaminergic neurons of the rat mesencephalon: an in vitro study. Funct Neurol 7, 231-234.

Mercuri, N.B., Stratta, F., Calabresi, P., Bonci, A. and Bernardi, G., 1993. Activation of metabotropic glutamate receptors induces an inward current in rat dopamine mesencephalic neurons. Neuroscience 56, 399-407.

Mereu, G., Lilliu, V., Casula, A., Vargiu, P.F., Diana, M., Musa, A. and Gessa, G.L., 1997. Spontaneous bursting activity of dopaminergic neurons in midbrain

slices from immature rats: role of N-methyl-D-aspartate receptors. Neuroscience 77, 1029-1036.

Miranda, M., Wu, C.C., Sorkina, T., Korstjens, D.R. and Sorkin, A., 2005. Enhanced ubiquitylation and accelerated degradation of the dopamine transporter mediated by protein kinase C. J Biol Chem 280, 35617-35624.

Mori, Y., Niidome, T., Fujita, Y., Mynlieff, M., Dirksen, R.T., Beam, K.G., Iwabe, N., Miyata, T., Furutama, D., Furuichi, T. and et al., 1993. Molecular diversity of voltage-dependent calcium channel. Ann N Y Acad Sci 707, 87-108.

Morikawa, H., Imani, F., Khodakhah, K. and Williams, J.T., 2000. Inositol 1,4,5-triphosphate-evoked responses in midbrain dopamine neurons. J Neurosci 20, RC103.

Morikawa, H., Khodakhah, K., Williams, J.T., 2003. Two intracellular pathways mediate metabotropic glutamate receptor-induced Ca2+ mobilization in dopamine neurons. J Neurosci 23, 149-157.

Nair-Roberts, R.G., Chatelain-Badie, S.D., Benson, E., White-Cooper, H., Bolam, J.P. and Ungless, M.A., 2008. Stereological estimates of dopaminergic, GABAergic and glutamatergic neurons in the ventral tegmental area, substantia nigra and retrorubral field in the rat. Neuroscience 152, 1024-1031.

Namkung, Y. and Sibley, D.R., 2004. Protein kinase C mediates phosphorylation, desensitization, and trafficking of the D2 dopamine receptor. J Biol Chem 279, 49533-49541.

Nastuk, M.A. and Graybiel, A.M., 1991. Pharmacologically defined M1 and M2 muscarinic cholinergic binding sites in the cat's substantia nigra: development and maturity. Brain Res Dev Brain Res 61, 1-10.

Nedergaard, S., Flatman, J.A. and Engberg, I., 1993. Nifedipine- and omega-conotoxin-sensitive Ca2+ conductances in guinea-pig substantia nigra pars compacta neurones. J Physiol 466, 727-747.

Nelson, E.L., Liang, C.L., Sinton, C.M. and German, D.C., 1996. Midbrain dopaminergic neurons in the mouse: computer-assisted mapping. J Comp Neurol 369, 361-371.

Neuhoff, H., Neu, A., Liss, B. and Roeper, J., 2002. I(h) channels contribute to the different functional properties of identified dopaminergic subpopulations in the midbrain. J Neurosci 22, 1290-1302.

Nisell, M., Nomikos, G.G. and Svensson, T.H., 1994. Systemic nicotine-induced dopamine release in the rat nucleus accumbens is regulated by nicotinic receptors in the ventral tegmental area. Synapse 16, 36-44.

Nowak, K.L., McBride, W.J., Lumeng, L., Li, T.K. and Murphy, J.M., 1998. Blocking GABA(A) receptors in the anterior ventral tegmental area attenuates ethanol intake of the alcohol-preferring P rat. Psychopharmacology (Berl) 139, 108-116.

Omelchenko, N. and Sesack, S.R., 2005. Laterodorsal tegmental projections to identified cell populations in the rat ventral tegmental area. J Comp Neurol 483, 217-235.

Osten, P., Valsamis, L., Harris, A. and Sacktor, T.C., 1996. Protein synthesis-dependent formation of protein kinase Mzeta in long-term potentiation. J Neurosci 16, 2444-2451.

Overton, P. and Clark, D., 1991. N-methyl-D-aspartate increases the excitability of nigrostriatal dopamine terminals. Eur J Pharmacol 201, 117-120.

Overton, P.G. and Clark, D., 1997. Burst firing in midbrain dopaminergic neurons. Brain Res Brain Res Rev 25, 312-334.

Overton, P.G., Tong, Z.Y. and Clark, D., 1996. A pharmacological analysis of the burst events induced in midbrain dopaminergic neurons by electrical stimulation of the prefrontal cortex in the rat. J Neural Transm 103, 523-540.

Paladini, C.A., Fiorillo, C.D., Morikawa, H. and Williams, J.T., 2001. Amphetamine selectively blocks inhibitory glutamate transmission in dopamine neurons. Nat Neurosci 4, 275-281.

Paladini, C.A. and Tepper, J.M., 1999. GABA(A) and GABA(B) antagonists differentially affect the firing pattern of substantia nigra dopaminergic neurons in vivo. Synapse 32, 165-176.

Paladini, C.A. and Williams, J.T., 2004. Noradrenergic inhibition of midbrain dopamine neurons. J Neurosci 24, 4568-4575.

Peloquin, J.B., Doering, C.J., Rehak, R. and McRory, J.E., 2008. Temperature dependence of Cav1.4 calcium channel gating. Neuroscience 151, 1066-1083.

Pezze, M.A. and Feldon, J., 2004. Mesolimbic dopaminergic pathways in fear conditioning. Prog Neurobiol 74, 301-320.

Picciotto, M.R., 1998. Common aspects of the action of nicotine and other drugs of abuse. Drug Alcohol Depend 51, 165-172.

Picciotto, M.R., Zoli, M., Rimondini, R., Lena, C., Marubio, L.M., Pich, E.M., Fuxe, K. and Changeux, J.P., 1998. Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. Nature 391, 173-177.

Pickel, V.M., Garzon, M. and Mengual, E., 2002. Electron microscopic immunolabeling of transporters and receptors identifies transmitter-specific functional sites envisioned in Cajal's neuron. Prog Brain Res 136, 145-155.

Pidoplichko, V.I., DeBiasi, M., Williams, J.T. and Dani, J.A., 1997. Nicotine activates and desensitizes midbrain dopamine neurons. Nature 390, 401-404.

Ping, H.X. and Shepard, P.D., 1996. Apamin-sensitive Ca(2+)-activated K+ channels regulate pacemaker activity in nigral dopamine neurons. Neuroreport 7, 809-814.

Pitts, D.K., Freeman, A.S. and Chiodo, L.A., 1990. Dopamine neuron ontogeny: electrophysiological studies. Synapse 6, 309-320.

Pitts, D.K., Kelland, M.D., Freeman, A.S. and Chiodo, L.A., 1993. Repeated amphetamine administration: role of forebrain in reduced responsiveness of

nigrostriatal dopamine neurons to dopamine agonists. J Pharmacol Exp Ther 264, 616-621.

Platzer, J., Engel, J., Schrott-Fischer, A., Stephan, K., Bova, S., Chen, H., Zheng, H. and Striessnig, J., 2000. Congenital deafness and sinoatrial node dysfunction in mice lacking class D L-type Ca2+ channels. Cell 102, 89-97.

Prensa, L. and Parent, A., 2001. The nigrostriatal pathway in the rat: A single-axon study of the relationship between dorsal and ventral tier nigral neurons and the striosome/matrix striatal compartments. J Neurosci 21, 7247-7260.

Prisco, S., Natoli, S., Bernardi, G., Mercuri, N.B., 2002. Group I metabotropic glutamate receptors activate burst firing in rat midbrain dopaminergic neurons. Neuropharmacology 42, 289-296.

Pucak, M.L. and Grace, A.A., 1994. Evidence that systemically administered dopamine antagonists activate dopamine neuron firing primarily by blockade of somatodendritic autoreceptors. J Pharmacol Exp Ther 271, 1181-1192.

Rada, P.V., Mark, G.P., Yeomans, J.J. and Hoebel, B.G., 2000. Acetylcholine release in ventral tegmental area by hypothalamic self-stimulation, eating, and drinking. Pharmacol Biochem Behav 65, 375-379.

Rajadhyaksha, A., Husson, I., Satpute, S.S., Kuppenbender, K.D., Ren, J.Q., Guerriero, R.M., Standaert, D.G. and Kosofsky, B.E., 2004. L-type Ca2+ channels mediate adaptation of extracellular signal-regulated kinase 1/2 phosphorylation in

the ventral tegmental area after chronic amphetamine treatment. J Neurosci 24, 7464-7476.

Robbins, T.W. and Everitt, B.J., 1999. Drug addiction: bad habits add up. Nature 398, 567-570.

Roberts, D.C., Koob, G.F., Klonoff, P. and Fibiger, H.C., 1980. Extinction and recovery of cocaine self-administration following 6-hydroxydopamine lesions of the nucleus accumbens. Pharmacol Biochem Behav 12, 781-787.

Romanides, A.J., Duffy, P. and Kalivas, P.W., 1999. Glutamatergic and dopaminergic afferents to the prefrontal cortex regulate spatial working memory in rats. Neuroscience 92, 97-106.

Sacktor, T.C., Osten, P., Valsamis, H., Jiang, X., Naik, M.U. and Sublette, E., 1993. Persistent activation of the zeta isoform of protein kinase C in the maintenance of long-term potentiation. Proc Natl Acad Sci U S A 90, 8342-8346.

Satoh, K. and Fibiger, H.C., 1986. Cholinergic neurons of the laterodorsal tegmental nucleus: efferent and afferent connections. J Comp Neurol 253, 277-302.

Sawaguchi, T. and Goldman-Rakic, P.S., 1994. The role of D1-dopamine receptor in working memory: local injections of dopamine antagonists into the prefrontal cortex of rhesus monkeys performing an oculomotor delayed-response task. J Neurophysiol 71, 515-528.

Schultz, W., 1997. Dopamine neurons and their role in reward mechanisms. Curr Opin Neurobiol 7, 191-197.

Schultz, W., 1998. Predictive reward signal of dopamine neurons. J Neurophysiol 80, 1-27.

Scroggs, R.S., Cardenas, C.G., Whittaker, J.A. and Kitai, S.T., 2001. Muscarine reduces calcium-dependent electrical activity in substantia nigra dopaminergic neurons. J Neurophysiol 86, 2966-2972.

Sculptoreanu, A., Yoshimura, N., de Groat, W.C. and Somogyi, G.T., 2001. Protein kinase C is involved in M1-muscarinic receptor-mediated facilitation of L-type Ca2+ channels in neurons of the major pelvic ganglion of the adult male rat. Neurochem Res 26, 933-942.

Seamans, J.K. and Yang, C.R., 2004. The principal features and mechanisms of dopamine modulation in the prefrontal cortex. Prog Neurobiol 74, 1-58.

Seeman, P., 1990. Atypical neuroleptics: role of multiple receptors, endogenous dopamine, and receptor linkage. Acta Psychiatr Scand Suppl 358, 14-20.

Seeman, P., Lee, T., Chau-Wong, M. and Wong, K., 1976. Antipsychotic drug doses and neuroleptic/dopamine receptors. Nature 261, 717-719.

Semba, K. and Fibiger, H.C., 1992. Afferent connections of the laterodorsal and the pedunculopontine tegmental nuclei in the rat: a retro- and antero-grade transport and immunohistochemical study. J Comp Neurol 323, 387-410.

Sesack, S.R., Carr, D.B., Omelchenko, N. and Pinto, A., 2003. Anatomical substrates for glutamate-dopamine interactions: evidence for specificity of connections and extrasynaptic actions. Ann N Y Acad Sci 1003, 36-52.

Seutin, V., Johnson, S.W. and North, R.A., 1993. Apamin increases NMDA-induced burst-firing of rat mesencephalic dopamine neurons. Brain Res 630, 341-344.

Seutin, V., Massotte, L., Renette, M.F. and Dresse, A., 2001. Evidence for a modulatory role of Ih on the firing of a subgroup of midbrain dopamine neurons. Neuroreport 12, 255-258.

Seutin, V., Mkahli, F., Massotte, L. and Dresse, A., 2000. Calcium release from internal stores is required for the generation of spontaneous hyperpolarizations in dopaminergic neurons of neonatal rats. J Neurophysiol 83, 192-197.

Severance, E.G. and Cuevas, J., 2004. Distribution and synaptic localization of nicotinic acetylcholine receptors containing a novel alpha7 subunit isoform in embryonic rat cortical neurons. Neurosci Lett 372, 104-109.

Sharma, G. and Vijayaraghavan, S., 2003. Modulation of presynaptic store calcium induces release of glutamate and postsynaptic firing. Neuron 38, 929-939.

Shea, T.B., Beermann, M.L., Griffin, W.R. and Leli, U., 1994. Degradation of protein kinase C alpha and its free catalytic subunit, protein kinase M, in intact human neuroblastoma cells and under cell-free conditions. Evidence that PKM is degraded by mM calpain-mediated proteolysis at a faster rate than PKC. FEBS Lett 350, 223-229.

Shepard, P.D. and Bunney, B.S., 1991. Repetitive firing properties of putative dopamine-containing neurons in vitro: regulation by an apamin-sensitive Ca(2+)-activated K + conductance. Exp Brain Res 86, 141-150.

Sinnegger-Brauns, M.J., Hetzenauer, A., Huber, I.G., Renstrom, E., Wietzorrek, G., Berjukov, S., Cavalli, M., Walter, D., Koschak, A., Waldschutz, R., Hering, S., Bova, S., Rorsman, P., Pongs, O., Singewald, N. and Striessnig, J.J., 2004. Isoform-specific regulation of mood behavior and pancreatic beta cell and cardiovascular function by L-type Ca 2+ channels. J Clin Invest 113, 1430-1439.

Smith, A.D. and Bolam, J.P., 1990. The neural network of the basal ganglia as revealed by the study of synaptic connections of identified neurones. Trends Neurosci 13, 259-265.

Somogyi, P., Bolam, J.P., Totterdell, S. and Smith, A.D., 1981. Monosynaptic input from the nucleus accumbens--ventral striatum region to retrogradely labelled nigrostriatal neurones. Brain Res 217, 245-263.

Sorenson, E.M., Shiroyama, T. and Kitai, S.T., 1998. Postsynaptic nicotinic receptors on dopaminergic neurons in the substantia nigra pars compacta of the rat. Neuroscience 87, 659-673.

Sorkina, T., Hoover, B.R., Zahniser, N.R. and Sorkin, A., 2005. Constitutive and protein kinase C-induced internalization of the dopamine transporter is mediated by a clathrin-dependent mechanism. Traffic 6, 157-170.

Steketee, J.D., 1993. Injection of the protein kinase inhibitor H7 into the A10 dopamine region blocks the acute responses to cocaine: behavioral and in vivo microdialysis studies. Neuropharmacology 32, 1289-1297.

Steketee, J.D., 1994. Intra-A10 injection of H7 blocks the development of sensitization to cocaine. Neuroreport 6, 69-72.

Steketee, J.D., 1997. Intra-ventral tegmental area administration of H7 delays, but does not prevent the development of cocaine-induced sensitization. Brain Res Bull 43, 565-571.

Steketee, J.D., Rowe, L.A. and Chandler, L.J., 1998. The effects of acute and repeated cocaine injections on protein kinase C activity and isoform levels in dopaminergic brain regions. Neuropharmacology 37, 339-347.

Stocker, M. and Pedarzani, P., 2000. Differential distribution of three Ca(2+)-activated K(+) channel subunits, SK1, SK2, and SK3, in the adult rat central nervous system. Mol Cell Neurosci 15, 476-493.

Striessnig, J., Koschak, A., Sinnegger-Brauns, M.J., Hetzenauer, A., Nguyen, N.K., Busquet, P., Pelster, G. and Singewald, N., 2006. Role of voltage-gated L-type Ca2+ channel isoforms for brain function. Biochem Soc Trans 34, 903-909.

Suaud-Chagny, M.F., Chergui, K., Chouvet, G. and Gonon, F., 1992. Relationship between dopamine release in the rat nucleus accumbens and the discharge activity of dopaminergic neurons during local in vivo application of amino acids in the ventral tegmental area. Neuroscience 49, 63-72.

Sugita, S., Johnson, S.W. and North, R.A., 1992. Synaptic inputs to GABAA and GABAB receptors originate from discrete afferent neurons. Neurosci Lett 134, 207-211.

Szallasi, Z., Smith, C.B., Pettit, G.R. and Blumberg, P.M., 1994. Differential regulation of protein kinase C isozymes by bryostatin 1 and phorbol 12-myristate 13-acetate in NIH 3T3 fibroblasts. J Biol Chem 269, 2118-2124.

Taber, M.T. and Fibiger, H.C., 1997. Activation of the mesocortical dopamine system by feeding: lack of a selective response to stress. Neuroscience 77, 295-298.

Takada, M., Kang, Y. and Imanishi, M., 2001. Immunohistochemical localization of voltage-gated calcium channels in substantia nigra dopamine neurons. Eur J Neurosci 13, 757-762.

Tapper, A.R., McKinney, S.L., Nashmi, R., Schwarz, J., Deshpande, P., Labarca, C., Whiteaker, P., Marks, M.J., Collins, A.C. and Lester, H.A., 2004. Nicotine activation of alpha4* receptors: sufficient for reward, tolerance, and sensitization. Science 306, 1029-1032.

Tarazi, F.I. and Baldessarini, R.J., 2000. Comparative postnatal development of dopamine D(1), D(2) and D(4) receptors in rat forebrain. Int J Dev Neurosci 18, 29-37.

Teicher, M.H., Barber, N.I., Gelbard, H.A., Gallitano, A.L., Campbell, A., Marsh, E. and Baldessarini, R.J., 1993. Developmental differences in acute nigrostriatal

and mesocorticolimbic system response to haloperidol. Neuropsychopharmacology 9, 147-156.

Tepper, J.M., Trent, F. and Nakamura, S., 1990. Postnatal development of the electrical activity of rat nigrostriatal dopaminergic neurons. Brain Res Dev Brain Res 54, 21-33.

Tobler, P.N., Fiorillo, C.D. and Schultz, W., 2005. Adaptive coding of reward value by dopamine neurons. Science 307, 1642-1645.

Tong, Z.Y., Overton, P.G. and Clark, D., 1996a. Antagonism of NMDA receptors but not AMPA/kainate receptors blocks bursting in dopaminergic neurons induced by electrical stimulation of the prefrontal cortex. J Neural Transm 103, 889-904.

Tong, Z.Y., Overton, P.G. and Clark, D., 1996b. Stimulation of the prefrontal cortex in the rat induces patterns of activity in midbrain dopaminergic neurons which resemble natural burst events. Synapse 22, 195-208.

Tsuji, M., Nakagawa, Y., Ishibashi, Y., Yoshii, T., Takashima, T., Shimada, M. and Suzuki, T., 1996. Activation of ventral tegmental GABAB receptors inhibits morphine-induced place preference in rats. Eur J Pharmacol 313, 169-173.

Ungerstedt, U., 1971. Stereotaxic mapping of the monoamine pathways in the rat brain. Acta Physiol Scand Suppl 367, 1-48.

Uramura, K., Funahashi, H., Muroya, S., Shioda, S., Takigawa, M. and Yada, T., 2001. Orexin-a activates phospholipase C- and protein kinase C-mediated Ca2+

signaling in dopamine neurons of the ventral tegmental area. Neuroreport 12, 1885-1889.

van der Heyden, M.A., Wijnhoven, T.J. and Opthof, T., 2005. Molecular aspects of adrenergic modulation of cardiac L-type Ca2+ channels. Cardiovasc Res 65, 28-39.

Vargas, G. and Lucero, M.T., 1999. Dopamine modulates inwardly rectifying hyperpolarization-activated current (Ih) in cultured rat olfactory receptor neurons. J Neurophysiol 81, 149-158.

Wang, H.S., Pan, Z., Shi, W., Brown, B.S., Wymore, R.S., Cohen, I.S., Dixon, J.E. and McKinnon, D., 1998. KCNQ2 and KCNQ3 potassium channel subunits: molecular correlates of the M-channel. Science 282, 1890-1893.

Wang, L. and Pitts, D.K., 1995. Ontogeny of nigrostriatal dopamine neuron autoreceptors: iontophoretic studies. J Pharmacol Exp Ther 272, 164-176.

Wang, T. and French, E.D., 1993a. Electrophysiological evidence for the existence of NMDA and non-NMDA receptors on rat ventral tegmental dopamine neurons. Synapse 13, 270-277.

Wang, T. and French, E.D., 1993b. L-glutamate excitation of A10 dopamine neurons is preferentially mediated by activation of NMDA receptors: extra- and intracellular electrophysiological studies in brain slices. Brain Res 627, 299-306.

Waszczak, B.L. and Walters, J.R., 1980. Intravenous GABA agonist administration stimulates firing of A10 dopaminergic neurons. Eur J Pharmacol 66, 141-144.

Watanabe, M., Kodama, T. and Hikosaka, K., 1997. Increase of extracellular dopamine in primate prefrontal cortex during a working memory task. J Neurophysiol 78, 2795-2798.

Watts, A.E., Williams, J.T. and Henderson, G., 1996. Baclofen inhibition of the hyperpolarization-activated cation current, Ih, in rat substantia nigra zona compacta neurons may be secondary to potassium current activation. J Neurophysiol 76, 2262-2270.

White, F.J. and Wang, R.Y., 1984. Pharmacological characterization of dopamine autoreceptors in the rat ventral tegmental area: microiontophoretic studies. J Pharmacol Exp Ther 231, 275-280.

Williams, J. and Lacey, M., 1988. Actions of cocaine on central monoamine neurons: intracellular recordings in vitro. NIDA Res Monogr 90, 234-242.

Wilson, C.J. and Callaway, J.C., 2000. Coupled oscillator model of the dopaminergic neuron of the substantia nigra. J Neurophysiol 83, 3084-3100.

Wolf, M.E. and Roth, R.H., 1987. Dopamine neurons projecting to the medial prefrontal cortex possess release-modulating autoreceptors. Neuropharmacology 26, 1053-1059.

Wolfart, J., Neuhoff, H., Franz, O. and Roeper, J., 2001. Differential expression of the small-conductance, calcium-activated potassium channel SK3 is critical for pacemaker control in dopaminergic midbrain neurons. J Neurosci 21, 3443-3456.

Wolfart, J. and Roeper, J., 2002. Selective coupling of T-type calcium channels to SK potassium channels prevents intrinsic bursting in dopaminergic midbrain neurons. J Neurosci 22, 3404-3413.

Wu, H.Q., Schwarcz, R. and Shepard, P.D., 1994. Excitatory amino acid-induced excitation of dopamine-containing neurons in the rat substantia nigra: modulation by kynurenic acid. Synapse 16, 219-230.

Wu, Y.N. and Johnson, S.W., 1996. Pharmacological characterization of inward current evoked by N-methyl-D-aspartate in dopamine neurons in the rat brain slice. J Pharmacol Exp Ther 279, 457-463.

Wu, Y.N., Shen, K.Z. and Johnson, S.W., 1999. Presynaptic inhibition preferentially reduces in NMDA receptor-mediated component of transmission in rat midbrain dopamine neurons. Br J Pharmacol 127, 1422-1430.

Xu, R., Roh, S.G., Gong, C., Hernandez, M., Ueta, Y. and Chen, C., 2003. Orexin-B augments voltage-gated L-type Ca(2+) current via protein kinase C-mediated signalling pathway in ovine somatotropes. Neuroendocrinology 77, 141-152.

Xu, W. and Lipscombe, D., 2001. Neuronal Ca(V)1.3alpha(1) L-type channels activate at relatively hyperpolarized membrane potentials and are incompletely inhibited by dihydropyridines. J Neurosci 21, 5944-5951.

Yamaguchi, T., Sheen, W. and Morales, M., 2007. Glutamatergic neurons are present in the rat ventral tegmental area. Eur J Neurosci 25, 106-118.

Yang, L., Liu, G., Zakharov, S.I., Morrow, J.P., Rybin, V.O., Steinberg, S.F. and Marx, S.O., 2005. Ser1928 is a common site for Cav1.2 phosphorylation by protein kinase C isoforms. J Biol Chem 280, 207-214.

Ye, J.H., Zhang, J., Xiao, C. and Kong, J.Q., 2006. Patch-clamp studies in the CNS illustrate a simple new method for obtaining viable neurons in rat brain slices: glycerol replacement of NaCl protects CNS neurons. J Neurosci Methods 158, 251-259.

Yoshihara, C., Saito, N., Taniyama, K. and Tanaka, C., 1991. Differential localization of four subspecies of protein kinase C in the rat striatum and substantia nigra. J Neurosci 11, 690-700.

Young, C.E. and Yang, C.R., 2004. Dopamine D1/D5 receptor modulates state-dependent switching of soma-dendritic Ca2+ potentials via differential protein kinase A and C activation in rat prefrontal cortical neurons. J Neurosci 24, 8-23.

Zahrt, J., Taylor, J.R., Mathew, R.G. and Arnsten, A.F., 1997. Supranormal stimulation of D1 dopamine receptors in the rodent prefrontal cortex impairs spatial working memory performance. J Neurosci 17, 8528-8535.

Zhang, H., Fu, Y., Altier, C., Platzer, J., Surmeier, D.J. and Bezprozvanny, I., 2006. Ca1.2 and CaV1.3 neuronal L-type calcium channels: differential targeting and signaling to pCREB. Eur J Neurosci 23, 2297-2310.

Zhang, J., Berra-Romani, R., Sinnegger-Brauns, M.J., Striessnig, J., Blaustein, M.P. and Matteson, D.R., 2007. Role of Cav1.2 L-type Ca^{2+} channels in vascular tone: effects of nifedipine and Mg^{2+}. Am J Physiol Heart Circ Physiol 292, H415-425.

Zhang, J., Chiodo, L.A. and Freeman, A.S., 1994. Influence of excitatory amino acid receptor subtypes on the electrophysiological activity of dopaminergic and nondopaminergic neurons in rat substantia nigra. J Pharmacol Exp Ther 269, 313-321.

Zhang, L., Liu, Y. and Chen, X., 2005. Carbachol induces burst firing of dopamine cells in the ventral tegmental area by promoting calcium entry through L-type channels in the rat. J Physiol 568, 469-481.

Zheng, F. and Johnson, S.W., 2002. Group I metabotropic glutamate receptor-mediated enhancement of dopamine cell burst firing in rat ventral tegmental area in vitro. Brain Res 948, 171-174.